**Humble beginnings**

*Trichoderma reesei* is a mesophilic filamentous fungus originally isolated from the Solomon Islands during the Second World War. The isolate was first identified as *Trichoderma viride* and named QM6a as part of the collection at the US Army QuarterMaster Research and Development Center at Natick, Massachusetts (Mandels & Reese, 1957). QM6a was later recognized as being distinct from *T. viride* and was given the new species name of *T. reesei* in honour of the Natick laboratory researcher Elwyn T. Reese (Simmons, 1977). *T. reesei* QM6a was found to be a good producer of cellulase (Mandels & Reese, 1957) and so its degradative action was soon seen as a highly valuable resource rather than a problem. The worldwide need for alternative fuel sources was becoming an increasing issue at the time and the potential of fungi to produce enzymes for the production of bioethanol (Eveleigh, 1982). The strain was obtained after three rounds of random mutagenesis of the wild-type QM6a in a screening program focused on high cellulase production and catabolite derepression. Whereas RUT-C30 achieves outstanding levels of protein secretion and high cellulolytic activity in comparison to the wild-type QM6a, recombinant protein production has been less successful. Here, we bring together and discuss the results from biochemical-, microscopic-, genomic-, transcriptomic-, glycomic- and proteomic-based research on the RUT-C30 strain published over the last 30 years.

**Breaking the mould – making high-cellulase-producing mutants**

The generation of high cellulase-producing mutants from the wild-type *T. reesei* QM6a was first attempted at Natick laboratories by irradiation of conidia using a linear accelerator (Mandels et al., 1971). The most successful mutant, QM9414, had an extracellular protein and cellulase production level two to four times that of QM6a, but remained catabolite-repressed (Mandels et al., 1971; Montenecourt & Eveleigh, 1977b). In a separate mutagenesis program developed at Rutgers University, New Jersey, a more efficient plate screening method was used in conjunction with UV and chemical mutagenesis to generate mutants from QM6a with higher cellulase activity than that previously achieved. The hypercellulolytic strain RUT-C30 was obtained through a three-step procedure (Montenecourt & Eveleigh, 1977a; Montenecourt & Eveleigh, 1977b, 1979). First, mutagenesis by UV light and screening for catabolite derepression led to the isolation of strain M7. Further mutagenesis by N-nitroguanidine led to the isolation of NG14, a partially derepressed strain that produced approximately twice the extracellular protein, five times the filter paper activity, twice the \( \beta \)-glucosidase activity and twice the endoglucanase activity of QM9414 (Montenecourt & Eveleigh, 1977b). Following another round of UV mutagenesis and screening for high cellulase activity and catabolite derepression by resistance to the antimetabolite 2-deoxyglucose (2DG), RUT-C30 was isolated (Montenecourt & Eveleigh, 1979).

RUT-C30 produced approximately 20 mg extracellular protein ml\(^{-1}\) and displayed cellulase activity of 15 filter paper units ml\(^{-1}\) under controlled fermenter conditions, a similar production level to that of NG14 and 15–20 times that of QM6a grown in shake flasks (Eveleigh, 1982; Bisaria & Ghose, 1981). In addition, unlike NG14 and its ancestors, RUT-C30 was catabolite-derpressed and displayed cellulase activity in the presence of 5% \( (v/v) \) glycerol that was almost equivalent to that produced on cellulose alone, and was also resistant to catabolite repression by 5% \( (w/v) \) glucose (Montenecourt & Eveleigh, 1979; Bisaria & Ghose, 1981). When grown on 6% \( (w/v) \) roll-milled cotton, the protein secretion of RUT-C30 was 2.7 times that of QM6a, and

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**Trichoderma reesei RUT-C30 – thirty years of strain improvement**

Robyn Peterson\(^1,2\) and Helena Nevalainen\(^1,2\)

\(^1\)Department of Chemistry and Biomolecular Sciences, Macquarie University, Australia
\(^2\)Biomolecular Frontiers Research Centre, Macquarie University, Australia

The hypersecreting mutant *Trichoderma reesei* RUT-C30 (ATCC 56765) is one of the most widely used strains of filamentous fungi for the production of cellulolytic enzymes and recombinant proteins, and for academic research. The strain was obtained after three rounds of random mutagenesis of the wild-type QM6a in a screening program focused on high cellulase production and catabolite derepression. Whereas RUT-C30 achieves outstanding levels of protein secretion and high cellulolytic activity in comparison to the wild-type QM6a, recombinant protein production has been less successful. Here, we bring together and discuss the results from biochemical-, microscopic-, genomic-, transcriptomic-, glycomic- and proteomic-based research on the RUT-C30 strain published over the last 30 years.
cellulase (filter paper) activity was 2.8 times that of QM6a, although β-glucosidase activities were similar (Table 1). RUT-C30 outperformed a high cellulase-producing mutant MCG77 (Gallo et al., 1978) and its parent strain QM9414. From that time to the present day, RUT-C30 has been used as a paradigm for developing processes for the production of cellulolytic enzymes (Olsson et al., 2003; Bailey & Táthiharju, 2003; Juhász et al., 2004; Singhania et al., 2007), engineering of hypercellulolytic strains (e.g. Collén et al., 2005; Limón et al., 2011) and production of cellulosases on agricultural and forest residues with a special emphasis of converting lignocellulosic biomass to ethanol (Gyalai-Korpos et al., 2011; Choy et al., 2011). Concurrently, it became apparent that the strain bears many mutations beyond cellulase secretion alone.

### Physiological changes in RUT-C30

The high protein secretion of *T. reesei* RUT-C30 was found to be associated with changes in the ultrastructural characteristics of the strain. Early electron microscopic analysis of RUT-C30 grown on Avicel cellulose exposed a six- to sevenfold higher endoplasmic reticulum (ER) content in RUT-C30 during secretion than in QM6a. Furthermore, the phospholipid content of mycelium and the amount of the ER marker enzyme nucleoside diphosphatase was approximately double that of QM6a on the fourth day of growth (Ghosh et al., 1982). Mycelial protein content of RUT-C30 was four–five times higher than that of QM6a, the endoglucanase activity was 30 times higher and β-glucosidase activity three times higher in RUT-C30 cells. RUT-C30 was also reported to lack typical Golgi bodies and instead contained many individual ER-associated saccules. Whether the saccules were in fact Golgi bodies with an unusual morphology or whether RUT-C30 was exhibiting a Golgi-independent secretory pathway that was not functional in QM6a remained undetermined (Ghosh et al., 1984; Glenn et al., 1985).

Our own recent studies into *T. reesei* ER using ultrastructural morphometry have revealed more details of the structural changes in the RUT-C30 hyphae. This high-protein-producing mutant seems to lack annulate lamellae abundant in the wild-type QM6a, and possess excessively transverse parallel cisternae and a small amount of punctate-like bodies, all indicating cellular stress (M. Nykänen and other authors, unpublished data). As for the strain phenotype, the conidia of RUT-C30 are of a lighter green colour and the bottom view of colonies growing on an agar plate lack the yellow colour (pigment) typical for the wild-type.

### Genetic changes in RUT-C30 detected in earlier studies

Since the *T. reesei* RUT-C30 strain was produced by random mutagenesis, the exact nature of the genetic basis underpinning the hyperproducing characteristics remained undetermined for over 20 years from its isolation. Classical genetic techniques were not applicable to study QM6a and its derivatives because the single original isolate and its descendants only reproduced asexually and the opposite mating type of QM6a had not yet been identified. Haploid recombinants could be obtained by means of protoplast fusion between different (mutant) strains, but the somatic diploid stage was too transient for successful hybridization studies and karyotyping by cytological methods (Manczinger & Ferenczy, 1985; Mäntylä et al., 1992).

With the advent of electrophoretic karyotyping, researchers from VTT and Alko laboratories in Finland (Mäntylä et al., 1992) and Genencor International in the USA (Carter et al., 1992) used contour-clamped homogeneous electric field (CHEF) gel electrophoresis to compare the genomes of QM6a, RUT-C30 and other hypercellulolytic strains. An increase in the size of chromosomes I–V and a decrease in size of chromosome VII was reported for RUT-C30 in comparison to QM6a, resulting in an overall increase in genome size from 32.5 to 34.7 Mbp (Carter et al., 1992). Gene mapping by Southern blot hybridization provided evidence for rearrangements between the chromosomes carrying genes encoding cellulolytic enzymes, including the

### Table 1. The extracellular protein and enzyme activities of wild-type *T. reesei* QM6a and mutants QM9414, MCG77, NG-14 and RUT-C30 following growth on 6 % (w/v) roll-milled cotton in a 10 l fermenter for 14 days (Ryu & Mandels, 1980)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soluble protein (mg ml⁻¹)</th>
<th>FPU (U ml⁻¹)</th>
<th>Productivity (FPU l⁻¹ h⁻¹)</th>
<th>CMC (U ml⁻¹)</th>
<th>βGl (U ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM6a</td>
<td>7</td>
<td>5</td>
<td>15</td>
<td>88</td>
<td>0.3</td>
</tr>
<tr>
<td>QM9414</td>
<td>14</td>
<td>10</td>
<td>30</td>
<td>109</td>
<td>0.6</td>
</tr>
<tr>
<td>MCG77*</td>
<td>16</td>
<td>11</td>
<td>33</td>
<td>104</td>
<td>0.9</td>
</tr>
<tr>
<td>NG-14</td>
<td>21</td>
<td>15</td>
<td>45</td>
<td>133</td>
<td>0.6</td>
</tr>
<tr>
<td>RUT-C30</td>
<td>19</td>
<td>14</td>
<td>42</td>
<td>150</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*MCG77 is a mutant strain derived by treating QM9414 with UV and kabacidin (Gallo et al., 1978; Persson et al., 1991).*
relocation of the main endoglucanase eglI to chromosome V in RUT-C30 from chromosome VI in QM6a (Mäntylä et al., 1992).

The genetic basis for the catabolite derepression exhibited by RUT-C30 became apparent when a glucose repressor gene creI was isolated. The gene was found to be truncated and encoded only one of the usual two zinc finger regions of the CREI protein (Ilmen et al., 1996). This mutation appeared to be specific to the RUT-C30 strain and was not found in the catabolite-repressed mutant QM9414 (Mandels et al., 1971). Cellulase mRNAs were produced by RUT-C30 (but not QM9414) on glucose-containing medium and transformation of the full-length creI gene into the strain restored glucose repression of cbhI expression (Ilmen et al., 1996). When mutants were constructed from T. reesei QM6a, by either completely removing the creI gene or replacing it with the truncated creI-1 from RUT-C30 (Nakari-Setälä et al., 2009), the mutants were derepressed in cellulase and hemicellulase production in the presence of glucose. All creI mutants also exhibited higher cellulase and hemicellulase activities than the wild-type when grown in a hydrodolase-inducing medium, indicating that the creI gene modulated both cellulase and hemicellulase gene expression in hydrodolase-inducing and non-inducing conditions.

Characterization of gene expression in RUT-C30 by transcriptomics

Transcriptional analysis of T. reesei RUT-C30 has further helped to characterize the enzymic activity of the mutant strain and detect possible genetic causes of bottlenecks in endogenous and heterologous protein secretion. Northern analysis was used to demonstrate that cellulase and hemicellulase genes were transcriptionally co-regulated under the control of the glucose repressor creI gene (Margolles-Clark et al., 1997). Thus cellulase and hemicellulase genes were expressed in the presence of glucose by RUT-C30, which carries the truncated creI gene (Ilmen et al., 1996), but not by QM9414 or RUT-C30 transformed with the wild-type creI gene. The expression of genes encoding cellulases and factors involved in protein folding and transport in RUT-C30 at different physiological states was also examined using Northern analysis, indicating higher cellubiohydrolase I (CBHI) synthesis and secretion at lower specific growth rates of fungal mycelia (Pakula et al., 2005). However, at low specific growth rates, the ratio of secreted to synthesized CBHI was lower and there was an upregulation of hacI, pdiI and bipI, genes associated with the unfolded protein response (UPR). The UPR pathway relieves cellular stress from accumulation of unfolded or misfolded proteins in the ER by increasing molecular chaperones and extra protein-folding-associated proteins, thereby enabling secretion or enhancing the rate of ER-associated degradation (Travers et al., 2000). Thus, the upregulation of UPR-associated genes in RUT-C30 as a response to the induction of high levels of CBHI production provided evidence that the strain was undergoing cellular stress influencing protein folding, transport and secretion.

Transcriptional analysis also revealed that recombinant protein production in RUT-C30 caused a greater upregulation of UPR-associated genes than endogenous cellulase production. The mRNA levels of bipI and pdiI in a RUT-C30 transformant expressing endoglucanase I (EGI) with a hydrophobic tag were upregulated to a slightly greater extent upon induction of cellulase genes by lactose than in the parent RUT-C30 strain (Collén et al., 2005). The induction of the UPR mediated by the hacI gene was also demonstrated in RUT-C30 under secretion stress by dithiothreitol (DTT) treatment (Saloheimo et al., 2003). Northern analysis and reverse transcription-PCR (RT-PCR) were used to reveal splicing of a 20 nt intron in the hacI gene upon UPR induction. The intron blocked transcription until splicing of the mRNA occurred, resulting in a change in the mRNA reading frame. Furthermore, transcriptional responses of RUT-C30 to secretion stress induced by DTT and recombinant protein production has been found to induce induction of genes for many ER- and secretion-related proteins that have been described in Saccharomyces cerevisiae, as well as upregulation of the cpcI transcription factor gene and nucleosomal genes (Arvas et al., 2006).

The transcriptional response to incorrectly folded proteins in T. reesei RUT-C30 was investigated in our laboratory using transformed RUT-C30 strains expressing mutant versions of the main secreted cellulase cellobiohydrolase I (CBHI) tagged with the fluorescent protein Venus (Kautto, 2009). Expression of the cbhI gene with four mutations resulted in massive upregulation of UPR-related genes such as bipI, lshI and cxlI, detected by microarray and qRT-PCR and produced physiological stress signs in the hyphae evidenced by confocal fluorescence microscopy (L. Kautto and other authors, unpublished data). Furthermore, a pulsing pattern in both transcription of the mutant CBHI mRNA and subsequent protein secretion was reported (Kautto, 2009). The pulsing pattern was also detected in the expression of native CBHI in RUT-C30 and in a RUT-C30 transformant strain expressing a heterologous bacterial xylanase gene, whereas the pulsing pattern in CBHI production in the wild-type QM6a was detectable but greatly reduced in comparison (M. Godlewski and other authors, unpublished data). There are indications that the pulsing pattern in secretion may be linked to the ER-to-Golgi maturation in these high-secreting strains. The ER-to-Golgi maturation may provide means for the morphological and functional adaptation of the secretory pathway to a substantial protein load in the high-secreting strains of T. reesei.

Sequencing pinpoints specific changes in the RUT-C30 genome

The steadily accumulating biochemical, molecular, transcriptomic and physiological evidence indicated that
RUT-C30 had undergone many more changes than an increase in cellulase production through the random mutagenesis program. Genome sequencing has now provided a global picture, whilst pinpointing exact changes that had occurred. The genome of T. reesei QM6a was fully sequenced in 2008 (Martínez et al., 2008). Soon to follow was the partial sequencing of RUT-C30 as part of an investigation of an apparently missing gene in the RUT-C30 strain (Seidl et al., 2008). Distinct genetic differences between the mutant and wild-type strains were revealed. An 85 kb genomic fragment was found to be missing in RUT-C30 and its ancestor NG14 (Seidl et al., 2008). The missing genes encoded enzymes concerned with primary metabolism (e.g. dehydrogenases, a trehalase and an aldo/keto reductase) and transporter proteins (e.g. a maltose permease, an aromatic amino acid permease and a monocarboxylate transporter), several extracellular enzymes (e.g. an imidase, a glucan endo-1,6-β-glucosidase and a rhamnogalacturonase) and proteins associated with cellular detoxification (a multidrug efflux pump and a glutathione S transferase).

Investigations were undertaken to link the phenotypic nature of RUT-C30 with the absent genes. The growth of RUT-C30 was impaired on α-linked oligo- and polysaccharides such as dextrin, starch and maltose, which was thought to be due the absence of the gene encoding maltose permease responsible for α-glucoside uptake. It was also observed that conidia of RUT-C30 swelled substantially in size before germinating, indicating impaired osmotic homeostasis that could have been a result of the missing glycerol dehydrogenase (GLD2) gene. The swelling phenomenon, not observed in QM9414, resulted in a prolonged lag phase in RUT-C30 conidia prior to forming mycelia. Some swollen conidia were not able to germinate and appeared to undergo autophagic cell death (Seidl et al., 2008). From this work it was evident that high cellulase production and catabolite repression were clearly not the only features of the RUT-C30 strain that differed from the wild-type. Other genetic and phenotypic alterations that could impact on overall cellular functioning also seemed to have occurred. Although only a small portion of the RUT-C30 genome was analysed in their work, Seidl et al. (2008) envisaged that further mutations and deletions would soon be discovered.

A year after the partial sequencing work by Seidl et al. (2008), massively parallel sequencing was used to compare the genomes of T. reesei RUT-C30 and its direct ancestor NG14 with the published genome of the wild-type QM6a (Le Crom et al., 2009). The strains were also assessed using phenotype microarrays for their ability to utilize different carbon sources. The genomes of RUT-C30 and NG14 were found to be missing over 100 kb of genomic DNA present in the wild-type QM6a, encompassing 18 large deletions [11 in NG14 including the 85 kb deletion previously identified by Seidl et al. (2008) and an additional seven in RUT-C30, including the known cre1 truncation previously identified by Ilmén et al. (1996)] and 15 small deletions or insertions (11 in NG14 and an additional four in RUT-C30). Furthermore, 223 single nucleotide variants (SNV) were detected (136 in NG14 and an additional 99 in RUT-C30, but 12 were eliminated as errors in the original QM6a sequence). The mutations and deletions affected 18 genes in NG14 and an additional 25 genes in RUT-C30 (Le Crom et al., 2009).

Newly discovered genes that were found to be mutated in both NG14 and RUT-C30 (Le Crom et al., 2009) included those with an assigned putative function of a vesicle transporter (MSF/major superfacilitator family), a kinesin-like Kif21a protein also involved in vesicle trafficking, a vacuolar sorting-associated protein and proteins involved in RNA metabolism and nuclear transport (Ataxin-7). The putative functions of additional genes that were mutated exclusively in RUT-C30 included a Golgi complex component (cog1) and a vacuolar ATPase (VSP1) with possible Golgi association and a subunit of a vacuolar ATPase, suggesting potential disruption of vacuolar protein sorting in RUT-C30. Genes related to yeast Ypt6 and Ypt7, activator proteins of Rab-like small GTPases were also mutated in RUT-C30. Small GTPases direct a variety of vesicle fusion events that influence internal membrane dynamics and the TOR (target of rapamycin) signalling pathway, which regulates cellular functions including gene transcription, translation and protein stability in fungal and other eukaryotic cells (Fernandes et al., 2005; Ralser et al., 2008; Singh & Tyers, 2009). The ypt6 gene encodes a Rab GTPase that is required for Golgi vesicle fusion and mutations in ypt6 in S. cerevisiae have resulted in inhibition of early Golgi function and ribosome biosynthesis (Li & Warner, 1996).

Also mutated exclusively in the RUT-C30 strain were three genes encoding nuclear transport proteins, potentially limiting transcription and signalling processes, and eight genes encoding proteins involved in metabolism, including those involved in sugar transport, a nitrlase or amidohydrolase, and an unknown esterase, lipase or thioesterase. Our own research (Peterson et al., 2011) has demonstrated significantly higher lipase activity from QM6a [5.9 nkat (mg secreted protein)⁻¹] than from RUT-C30 [0.1 nkat (mg secreted protein)⁻¹], consistent with the mutation in the lipase gene. A mutation in a maltose permease gene was also detected (Le Crom et al., 2009) in addition to the maltose permease lost in RUT-C30 in the 85 kb deletion (Seidl et al., 2008). Correspondingly, the utilization of α-linked oligosaccharides and glycans by RUT-C30, as assessed by phenotype microarray, was substantially different to that of QM6a (Le Crom et al., 2009). Furthermore, our own research has demonstrated far greater levels of amylase activity from QM6a [320 nkat (mg secreted protein)⁻¹] than from RUT-C30 [8 nkat (mg secreted protein)⁻¹] (Peterson et al., 2011). In contrast, results from phenotype microarray (Le Crom et al., 2009) indicated that the utilization of catabolite-repressing carbon sources such as glucose, fructose, mannose and
D-xylose was increased in RUT-C30, corresponding to the mutation of the glucose repressor gene, creI.

The genetic comparisons of the mutant RUT-C30 with the wild-type QM6a revealed massive changes in the T. reesei genome caused by the random mutagenesis and screening program used for the generation of high cellulosic strains (Montenecourt & Eveleigh, 1977a, b, 1979). Evidence for mutation and deletion of genes involved in protein production and secretion and other major cellular functions could now help to explain biochemical, morphological and physiological differences discovered between the strains from past or future research.

**The discovery of alterations to protein glycosylation in RUT-C30**

Glycomic analysis provided evidence that the extracellular hydrolases of RUT-C30 had atypical glycosylation profiles not exhibited by the wild-type QM6a. The N-glycans on the main secreted cellulase of T. reesei RUT-C30, CBH, were found to carry an unusual 1,3-glucose residue (De Bruyn et al., 1997; Maras et al., 1997a; Hui et al., 2001; Stals et al., 2004), indicating incomplete glycan processing. The unusual glycosylation profile was also found on CBHI secreted by T. reesei RL-P37, a mutant strain derived from NG14 from which RUT-C30 was obtained. Both RUT-C30 and RL-P37 were selected for catabolite derepression by their resistance to 2DG (Montenecourt & Eveleigh, 1979), a known glycosylation inhibitor (Datema & Schwarz, 1978; Hubbard & Ivatt, 1981), indicating that the use of 2DG in the selection process may have resulted in the isolation of mutants with atypical glycosylation profiles in comparison to the wild-type strain QM6a. The acetylxylan esterase from RUT-C30 was also found to exhibit an unusual glycosylation profile in which the N-glycan was both phosphorylated and highly mannosylated, and the linker peptide was heavily O-glycosylated and also sulphated (Harrison et al., 2002). In comparison, CBHI of T. reesei QM9414, a strain from a separate mutation line derived from NG14 was found to be very similar to that of RUT-C30, whereas N-glycans from QM9414 were significantly different and were not monoglucosylated, a finding consistent with previous research (Garcia et al., 2001; Stals et al., 2004). Consequently, the abnormal glycosylation of secreted proteins resulting from the mutant gls2 was confirmed as being specific to RUT-C30, its parent strain NG14 and probably other strains derived from it, such as RL-P37 (Stals et al., 2004; Geysens et al., 2005).

**Proteomics of RUT-C30**

Proteomic tools, such as 1D and 2D gel electrophoresis and MS have been widely used to investigate the secreted and/or intracellular proteins produced by filamentous fungi (reviewed by Carberry & Doyle, 2007; Kim et al., 2007; Gonzalez-Fernández et al., 2010), yet direct comparisons of the proteome of RUT-C30 and QM6a have, to our knowledge, not been reported to date. However, the secretomes of RUT-C30 and another high-cellulase-producing mutant T. reesei CL847 derived from QM9414 have been compared (Herpoël-Gimbert et al., 2008). The secretome of RUT-C30 grown on lactose as a sole carbon source was notably dominated by a high proportion of CBHI, whereas the secretome of CL847 was more diverse with less CBHI but higher levels of β-glucosidase, mannanase and xylan-related enzymes. The heavy dominance of CBHI was also reported from MS/MS-based ‘shotgun’ proteomic analysis of the secretome of RUT-C30 grown on corn stover (Nagendran et al., 2009). The identity of six-ten extracellular proteases in the RUT-C30 secretome was also revealed. Although RUT-C30 is recognized as a low protease mutant in comparison with the wild-type QM6a (Sheir-Neiss & Montenecourt, 1984; Peterson et al., 2011), the continued presence of proteases that can degrade endogenous and heterologous proteins remains an issue and is currently being investigated in our laboratory using proteomic analysis.

**RUT-C30 as a host for heterologous protein production**

The production of heterologous proteins in T. reesei RUT-C30 has been only moderately successful to date (Table 2) and considerably lower yields are achieved than for native proteins that have been reported to be produced at up to 100 g l⁻¹ in controlled fermenter cultivations (Cherry & Fidantsef, 2003). The traditional method of transformation of T. reesei is protoplast fusion (Penttilä et al., 1987). However, the technique has been particularly difficult with RUT-C30, seemingly due to a reduced ability of the strain to regenerate its cell wall (Nevalainen et al., 1995; Bergquist et al., 2002). Biolistic bombardment has provided a quick and reliable transformation system of T. reesei in general, whereby a good number of stable transformants are generated (Hazzell et al., 2000; Te’o et al., 2002). However, despite several attempts at optimization, including modification of codon usage of heterologous genes for
fungal expression (Te’o et al., 2000), heterologous protein production has not been reported to exceed 2 g l⁻¹ in the published literature. Amongst the mechanisms that have been proposed to cause limitations to heterologous protein production in RUT-C30 are the UPR and ER-associated degradation of the foreign gene product (Colle´n et al., 2005; Arvas et al., 2006; Kautto and other authors, unpublished data).

Microscopy has been used to visually identify bottlenecks along the secretory pathway of heterologous proteins. For example, the expression and secretion of homologous CBHI and a heterologous barley endopeptidase (EPB) in a RUT-C30-based transformant (Saarelainen et al., 1997) was studied using in situ hybridization, indirect immunofluorescence and immunoelectron microscopy (Nyka¨nen et al., 1997). Recombinant EPB appeared trapped in the ER and in spherical vesicles in the apical regions, whereas both cbh1 mRNA and CBHI were distributed throughout the hyphae and particularly localized close to the plasma membrane in elongated vesicles and in the cell walls (Nyka¨nen et al., 1997). Fluorescence and immunoelectron microscopy have also been used to reveal co-localization of incorrectly folded proteins with the proteasome, the centre for protein degradation in the cell cytoplasm (Kautto, 2009). However, although considerable research has been devoted to determining the reasons for low heterologous protein production in RUT-C30, the exact causes remain elusive.

**Where from now?**

Homologous recombination has been the major approach for producing knockout strains in T. reesei with varying levels of success (reviewed by Ruiz-Diez, 2002; Meyer, 2008). Recent advances in method development have resulted in high-throughput generation of knockout/gene replacement strains by more efficient strategies, increasing the efficiency of homologous recombination and allowing sequential gene deletions. While these strategies have so far been applied to T. reesei QM9414-derived strains (Hartl & Seiboth, 2005; Guangtao et al., 2009) and the wild-type QM6a (Steiger et al., 2011), they could similarly be applied to the RUT-C30 strain. In addition, alternative strategies such as gene knock-down by mRNA silencing (reviewed by Nakayashiki et al., 2005) could be employed for partial or condition-specific suppression of gene expression, particularly when gene deletion could be detrimental or even lethal. The use of RNA silencing in T. reesei is in its infancy but shows potential for future development. For example, the expression of the xylitol dehydrogenase gene xdh1 from

### Table 2. Production of heterologous proteins in T. reesei RUT-C30 and other mutant T. reesei strains

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Heterologous protein</th>
<th>Culture conditions</th>
<th>Production level (g l⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUT-C30</td>
<td>Calf chymosin</td>
<td>Fermenter (10 l)</td>
<td>0.04</td>
<td>Harkki et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Glucoamylase P from</td>
<td>Shake flask (50 ml)</td>
<td>0.5</td>
<td>Joutsjoki et al. (1993a, b)</td>
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<tr>
<td></td>
<td>Hormoncon resinae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBHI-Fab fusion antibody</td>
<td>Shake flask</td>
<td>0.04</td>
<td>Nyyssönen et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>Endochitinase from</td>
<td>Bioreactor</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trichoderma harzianum</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Barley endopeptidase B</td>
<td>Shake flask (50 ml)</td>
<td>0.13</td>
<td>Margolles-Clark et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>β-Glucosidase from</td>
<td>Shake flask (50 ml)</td>
<td>0.0027</td>
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<td></td>
<td>Talaronycys emersonii</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Laccase from Melanocarpus albonycyces</td>
<td>Fermenter (20 l)</td>
<td>0.23</td>
<td>Kiiskinen et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>XynVI from Acrophialophora nutiniana</td>
<td>Shake flask (50 ml)</td>
<td>0.172</td>
<td>Salles et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Cinnamoyly esterase EstA from</td>
<td>Shake flask (50 ml)</td>
<td>0.033</td>
<td>Poidevin et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>Hormoncon resinae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALKO2221*</td>
<td>Glucoamylase P from</td>
<td>Shake flask (50 ml)</td>
<td>0.7</td>
<td>Joutsjoki et al. (1993a, b)</td>
</tr>
<tr>
<td></td>
<td>Acid phosphatase from</td>
<td>Shake flask (50 ml)</td>
<td>0.5</td>
<td>Miettinen-Oinonen et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Aspergillus niger</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALKO3620*</td>
<td>Barley endopeptidase B</td>
<td>Fermenter (1 l)</td>
<td>0.5</td>
<td>Saarelainen et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Xyn11A from Nonomuraea flexuosa</td>
<td>Fermenter (1 l)</td>
<td>0.82</td>
<td>Paloheimo et al. (2003)</td>
</tr>
<tr>
<td>HEP1*</td>
<td>Xylanase II from Humicola grisea</td>
<td>Fermenter (2 l)</td>
<td>1.9</td>
<td>Paloheimo et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shake flask (50 ml)</td>
<td>0.5</td>
<td>de Faria et al. (2002)</td>
</tr>
</tbody>
</table>

*ALKO2221, ALKO3620 and HEP1 are strains derived from QM9414 following one or more rounds of mutation or transformation.
RUT-C30 was inhibited using an antisense construct resulting in a fivefold increase in xylitol production (Wang et al., 2005). In addition, suppression of the CBHII (CEL6A) gene, encoding the highly secreted cellulohydrolase II, was achieved in T. reesei RUT-C30 (Brody & Maiyuran, 2009) using RNA interference (RNAi). It is possible that reducing the level of CBHII expression in a similar manner may relieve the cell from the stresses on protein folding and secretion that are usually imposed by the production of the highly secreted cellulase and make the cellular machinery more amenable for recombinant protein production.

The production of fully functional heterologous proteins of mammalian origin requires more complex glycosylation pathways than those naturally occurring in filamentous fungi. Correct glycan additions are required in order for the proteins to be fully active and to avoid immunogenic responses when used as therapeutic pharmaceuticals (reviewed by Gerngross, 2004; Lubertozzi & Keasling, 2009). Attempts made to modify the secreted high mannose-type glycoproteins from RUT-C30 to the complex mammalian type by in vitro processing with mammalian glycosyltransferases have only been moderately successful (Maras et al., 1997b, 1999). Yields were low and the processing was costly and time consuming. Genetic engineering of a humanized glycosylation pathway may be desirable for a fungal host designed to produce mammalian proteins of therapeutic value. However, the task is immense and is yet to be fully achieved with filamentous fungi (Gerngross, 2004; Lubertozzi & Keasling, 2009). The drawback of this approach is that genetic engineering of the glycosylation enzymes will have an effect on all glycoproteins expressed in the production host and will thereby impede cell viability.

A more targeted approach for the production of a fungal host for recombinant protein production would result in a strain with known genetic changes rather than a random number of mutations that would only be discovered by full genome analysis. The importance of assessing the global effect of mutations is becoming increasingly apparent. The complete sequencing of the T. reesei QM6a genome makes the wild-type strain suitable for targeted metabolic engineering strategies for both improved cellulase production (reviewed by Kubicek et al., 2009) and heterologous protein production (reviewed by Boghigian et al., 2010; Matsuoka & Shimizu, 2010; Melzer et al., 2009). Metabolic modelling and flux analysis, in which the flow of carbon and nitrogen can be tracked through a metabolic network, may provide insight into bottlenecks along metabolic pathways that cause reduced yields. Quantitative metabolic flux analysis utilizing $^{13}$C labelling has recently been used to compare the biosynthetic pathways of amino acids in T. reesei QM6a and a crel deletion strain (Jouhten et al., 2009). Although metabolic modelling has not yet been fully applied to heterologous protein production in T. reesei, successes have been achieved in S. cerevisiae for the production of a heterologous cubebol, a natural sesquiterpene alcohol (Asadollahi et al., 2009). Minimization of metabolic adjustments required for heterologous cubebol production was achieved by appropriate gene deletions and gene overexpressions, resulting in an 85% improvement in yield.

The increasing number of sequenced genomes provides a convenient pool of gene products that can be expressed in selected hosts as either the major or supplementary product, or for a particular purpose of ‘fixing a problem’, e.g. to facilitate correct post-translational modifications, protein folding and secretion. In addition, the value of screening fungal species from the environment to search for new protein products and/or new potential high secreting hosts cannot be overlooked (Peterson et al., 2011). The benefits from returning to the natural environment are well illustrated by the recent discovery of the mating type counterpart of QM6a amongst natural isolates of Hypocrea jecorina (Seidl et al., 2009), which has opened up a previously unexplored, yet more traditional, avenue for combining different strain properties via sexual crossing. For over 50 years since its isolation, the wild-type T. reesei QM6a was considered to exist only in an asexual (anamorphic) form. However, sexual reproduction of the species has now been achieved between QM6a, identified as having a MAT1-2 mating type locus, and other wild-type strains carrying the opposite MAT1-1 locus. Furthermore, sexual reproduction was also achieved between MAT1-1 strains and mutant strains, including RUT-C30 and QM9414 (Seidl et al., 2009). Sexual crossing can therefore now be used as a fast and efficient method of eliminating detrimental mutations by complementation with the wild-type and combining favourable features expressed by different successful mutant strains.

**Conclusion**

T. reesei RUT-C30, a hypercellulolytic strain, was isolated in 1979 and has since become a household name in both academic and industry-driven studies into lignocellulose-degrading enzymes and their applications. While several studies treat RUT-C30 as a strain that produces an increased amount of cellulates, especially CBHII, it is important to note that the genome of RUT-C30 has undergone a great number of changes from the wild-type QM6a, in addition to the higher cellulase activity for which it was originally selected. Some of the described changes influence the overall cellular function and organelle structure such as the ER; thus, using the RUT-C30 strain to draw conclusions about T. reesei in general must be done with caution. While application of the -omics technologies to studies of T. reesei RUT-C30 have shed light on the molecular basis of some of the changes, the genetic basis for increased cellulase production is still not evident. RUT-C30 has been used as a host for recombinant protein production with varying levels of success. Increasing the levels of recombinant gene products may be dependent on advancement of the knowledge of gene regulation and...
protein quality control mechanisms operating in *T. reesei*. Finally, with the current emphasis on the development of processes for enzymatic conversion of lignocellulosic biomass to ethanol, the circle seems complete; the high-cellulase-producing RUT-C30 was originally created to combat fuel shortage caused by the oil crisis 30 years ago.

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References


