Engineering filamentous fungi for the conversion of D-galacturonic acid to L-galactonic acid

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Abstract

D-galacturonic acid, the main monomer of pectin, is an attractive substrate for bioconversions, since pectin rich biomass is abundantly available and pectin is easily hydrolysed. L-Galactonic acid is an intermediate in the eukaryotic pathway for D-galacturonic acid catabolism, but extracellular accumulation of L-galactonic acid has not been reported. By deleting the gene encoding L-galactonic acid dehydratase (lgd1 or gaaB) in two filamentous fungi, strains were obtained that converted D-galacturonic acid to L-galactonic acid. Both Trichoderma reesei Δlgd1 and Aspergillus niger ΔgaaB produced L-galactonate at yields of 0.6 to 0.9 g per g substrate consumed. While T. reesei Δlgd1 could produce L-galactonate at pH 5.5, lower pH was necessary for A. niger ΔgaaB. Provision of a co-substrate improved the production rate and titre in both strains. Intracellular accumulation of L-galactonate (40 to 70 mg [g biomass]⁻¹) suggested that export may be limiting. Deletion of the L-galactonate dehydratase from A. niger was found to delay induction of D-galacturonate reductase and overexpression of the reductase improved initial production rates. Deletion of the L-galactonate dehydratase from A. niger also delayed or prevented induction of the putative D-galacturonate transporter An14g04280. In addition, A. niger ΔgaaB produced L-galactonate from polygalacturonate as efficiently as from the monomer.

Introduction

D-galacturonic acid is the principal component of pectin, a major constituent of sugar beet pulp and citrus peel which are abundant and inexpensive raw materials. The annual worldwide production of sugar beet and citrus fruit is about 250 x 10⁶ and 115 x 10⁶ metric tons respectively. After beet processing, 5-10% of the sugar beet remains as dried sugar beet pulp. This pulp contains about 25% pectin (5). Citrus peel contains about 20% pectin on a dry mass basis. Sugar beet pulp and citrus peel are mainly used as cattle feed or they are dumped. The use
as cattle feed requires that pulp and peel are dried since otherwise they rot rapidly. Disposal of
the material is problematic because of the bad odour generated at the dumping sites. In the case
of sugar beet pulp the energy consumption for drying and pelleting are 30% to 40% of the total
energy used for beet processing (5). This process is only economical when done in large scale
and when energy costs are low. Other products, such as pectin and limonene, may be extracted
from citrus peel. Pectin is used as a gelling agent in the food industry; limonene as a flavour
compound. These are limited markets and with increasing energy costs and alternative animal
feed sources reducing the revenues from pectin-rich biomass for cattle feed sales, it is desirable
to find new ways to convert this biomass to other useful products. This may be accomplished by
microbial fermentation (16). Genetically modified bacteria have been used to produce ethanol
from pectin rich biomass (6, 7). Using genetically modified fungi, D-galacturonic acid has been
converted to galactaric acid (14) or to 2-keto-3-deoxy-L-galactonic acid (20).

Using fungi to valorise D-galacturonic acid is attractive since many species can use D-
galacturonic acid efficiently for growth, indicating that these species have efficient D-
galacturonic acid uptake. Filamentous fungi, especially Aspergillus niger, may also efficiently
produce pectinases, enabling simultaneous hydrolysis and conversion of the pectin rich biomass.
Other advantages are that many fungi are robust, low pH tolerant organisms with simple
nutritional requirements.

In fungi, D-galacturonic acid is catabolised through a pathway (Fig. 1) which includes
reactions catalysed by D-galacturonic acid reductase (10), L-galactonate dehydratase (9), 2-keto-
3-deoxy galactonate aldolase (8) and L-glyceraldehyde reductase (11); the intermediates are L-
galactonate, 2-keto-3-deoxy-L-galactonate (3-deoxy-L-threo-hex-2-ulosonate) and L-
glyceraldehyde and the products of the pathway are pyruvate and glycerol. D-galacturonic acid
can induce pectinolytic and D-galacturonic acid catabolic genes in *A. niger*, regardless of whether D-galacturonic acid is metabolised or not (4, 14).

By disrupting the native D-galacturonic acid catabolic pathway it is possible to engineer fungal strains for alternative D-galacturonic acid conversions (14, 20). In the case of galactaric acid production, the gene encoding D-galacturonic acid reductase was deleted and a gene encoding a D-galacturonic acid dehydrogenase expressed (14). Strains lacking the reductase were unable to grow on D-galacturonic acid and the strains also expressing the dehydrogenase converted D-galacturonic acid to galactaric acid. To produce 2-keto-3-deoxy-L-galactonic acid, it was only necessary to delete the gene for the 2-keto-3-deoxy-L-galactonic acid aldolase (20). The resulting strain did not grow on D-galacturonic acid (8), but converted D-galacturonic acid to 2-keto-3-deoxy-L-galactonic acid. The pathway for D-galacturonic acid catabolism in fungi can also be interrupted at the L-galactonate dehydratase step. A strain of *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) in which the L-galactonate dehydratase, lgd1, was deleted was unable to grow on D-galacturonic acid (9). In the present communication we show that deletion of the gene encoding L-galactonate dehydratase, i.e. *lgd1* in *T. reesei* and gaaB in *A. niger*, results in strains that convert D-galacturonic acid to L-galactonic acid which is excreted into the medium.

L-galactonic acid is currently expensive and not widely used, but has the potential to be used more widely once it is available at a low price. The physico-chemical properties are similar to those of D-gluconic acid, which is widely used as a chelator, in the pharmaceutical, cosmetic, and other industrial (e.g. dyes, detergents, solvents, paints) sectors and as an acidifier in food. L-Galactonic acid is also a precursor for L-ascorbic acid (vitamin C) synthesis. The L-galactono-
1,4-lactone which forms from L-galactonic acid at acidic pH can be oxidised to L-ascorbic acid chemically (3) or in a fermentative process (17).

Materials and Methods

Strains. The deletion of the \textit{lgd1} in \textit{Trichoderma reesei} (anamorph of \textit{Hypocrea jecorina}) was described previously (9).

\textit{Aspergillus niger} ATCC 1015 \textit{ΔpyrG}, with the gene encoding the orotidine-5’-phosphate decarboxylase (\textit{pyrG}) deleted (14), was used to construct the \textit{gaaB} deletion strain. The cassette for deletion of \textit{gaaB} contained 1550 bp from the \textit{A. niger gaaB} promoter, 1533 bp from the \textit{A. niger gaaB} terminator, and a 1920 bp fragment containing the \textit{pyrG} gene flanked with its native promoter and terminator. These fragments were obtained by PCR of \textit{A. niger} ATCC1015 genomic DNA using primers gaaB-5-F, gaaB-5-R, gaaB-3-F, gaaB-3-R, \textit{pyrG}-del-F\textunderscore n, and \textit{pyrG}-del-R\textunderscore n (Table 1), and the proofreading DNA polymerase Phusion (Finnzymes). Plasmid pRSET-A (Invitrogen) was digested with \textit{EcoRI} and \textit{PvuII} (both NEB) and the terminator fragment (\textit{gaaB}-3) with \textit{EcoRI} to produce an intermediary construct by ligation using T4 DNA ligase (NEB). This intermediary construct was digested with \textit{Xhol} (NEB) and \textit{Ecl136II} (Fermentas) and ligated to the \textit{Xhol} digested promoter fragment (\textit{gaaB}-5). The resulting vector was digested with \textit{Ecl136II} and treated with phosphatase. The \textit{pyrG} DNA fragment, after digestion with \textit{SmaI}, was inserted between the two \textit{gaaB} flanking regions. The deletion cassette, 5006 bp containing the \textit{gaaB} flanking regions and the \textit{pyrG} gene, was released by \textit{EcoRI} + \textit{Xhol} digestion and introduced into \textit{A. niger} ATCC1015 \textit{ΔpyrG} as described previously (14). Transformants were selected by ability to grow in the absence of uracil. Strains with a correct deletion were verified by PCR and tested for growth on D-galacturonate as a sole carbon source.
The cassette for the overexpression of *A. niger* D-galacturonate reductase (*gaaA*) contained the native *gaaA* gene between the *gpdA* promoter and *trpC* terminator from *A. nidulans*, following the hygromycin B phosphotransferase (*hph*) gene under the *gpdA* promoter. The *gaaA* fragment was obtained by PCR from ATCC1015 genomic DNA using the primers *gaaA-exp-F* and *gaaA-exp-R* (Table 1). The plasmid (JKp1-*hph*) containing the *gpdA-trpC-hph* fragment was derived from pRS426 (ATCC). Both JKp1-*hph* and the PCR-amplified *gaaA* fragment were digested with *SacI* and *XmaI* (both NEB), followed by ligation using T4 DNA ligase to generate the intermediary construct JKp1-*hph-gaaA*. JKp1-*hph-gaaA* was digested with *BspHI* and *PsiI* (both NEB) and the fragment containing the *gpdA-gaaA-trpC-hph* cassette was introduced into *A. niger* ATCC1015 *gaaBΔ* strain by transformation. Transformants were screened for integration of the *gpdA-gaaA-trpC-hph* cassette by growth in the presence of 400 μg ml⁻¹ hygromycin B (Calbiochem). Integration of the transformed cassette into the genome was confirmed by PCR with the primers *gpdA-F* and *gaaA-exp-R* (Table 1).

**Media.** The defined medium of Vogel (19), modified as described by Mojzita et al. (14), was used to assess L-galactonate production in flasks and bioreactors. D-Xylose (2 to 11 g l⁻¹) was provided as carbon source and ammonium sulphate (1.65 or 3.3 g l⁻¹) as nitrogen source. D-galacturonate (approximately 10 g l⁻¹; prepared as sodium salt), or polygalacturonate (15 g l⁻¹; prepared as sodium salt and containing 11 g D-galacturonic acid l⁻¹ plus 1 g l⁻¹ combined D-xylose, D-galactose and D-mannose when hydrolysed) were used as substrates in production media. Alternatively, the *A. nidulans* defined minimal medium of Barratt et al. (1) was used for *A. niger* cultures with 20 g D-galacturonate l⁻¹ and 5 g D-xylose l⁻¹. The pH of production media was adjusted between 3.0 and 6.0 with NaOH.
Medium (modified from Vogel (19)) for pre-cultures contained 20 g xylose l\(^{-1}\) and was supplemented with 1 g bactopeptone l\(^{-1}\) to provide more rapid growth in this chemically defined medium. \textit{A. niger} pre-cultures also contained 4 g agar l\(^{-1}\) or 30 g gelatin l\(^{-1}\), so that growth would be more filamentous. Agar was used in pre-cultures for bioreactor cultures, since it was not metabolised by \textit{A. niger}, and thus the biomass received the same nutrients as the \textit{T. reesei} precultures. For studies of gene expression, pre-cultures of \textit{A. niger} were grown in medium containing 10 g yeast extract l\(^{-1}\), 20 g peptone l\(^{-1}\) (YP) and 30 g gelatin l\(^{-1}\).

**Cultural conditions.** Small scale cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium and incubated at 30°C, 200 rpm. Pre-culture flasks were inoculated with conidial suspensions (final concentrations 5.3 x 10\(^5\) conidia ml\(^{-1}\)) and production flasks with mycelium from the pre-cultures. \textit{T. reesei} pre-cultures were allowed to grow for approximately 24 h before being harvested by vacuum filtration through disks of sterile, disposable cleaning cloth (X-tra, 100% viscose household cleaning cloth, Inex Partners Oy, Helsinki) and rinsed with sterile H\(_2\)O (> 2 volumes) to remove residual peptone and D-xylose. \textit{A. niger} was grown for 24 h in pre-culture medium containing 4 g agar l\(^{-1}\) or 30 g gelatin l\(^{-1}\) to reduce formation of pellets. Mycelium (5 ml) from agar-containing pre-cultures was transferred to fresh pre-culture medium lacking agar (50 ml) and incubated for 18 h to reduce the agar content in the cultures and provide inoculum consisting of very small (<2 mm diam.) pellets for D-galacturonate conversion which could be filtered and washed in the same manner as the \textit{T. reesei} pre-cultures. Alternatively, gelatin-containing pre-cultures were harvested by vacuum filtration and rinsed with sterile H\(_2\)O warmed to 37 °C to remove gelatin, then with cold H\(_2\)O. Washed mycelium was aseptically transferred to production medium.
For larger scale cultures, mycelium was grown in bioreactors in 500 ml (Multifors, max working volume 500 ml, Infors HT, Switzerland). Cultures were maintained at 30°C, 800 rpm, with 1.6 volume gas [volume culture]⁻¹ min⁻¹ (vvm). Culture pH was kept constant at pH 4.5, 4.9 or 5.5 by the addition of sterile 1 M KOH or 1 M H₃PO₄. Polypropylene glycol (mixed molecular weight (21)) was added to control foam production.

The initial biomass concentration in *T. reesei* cultures was 0.3 g l⁻¹ and in *A. niger* cultures 0.4 g l⁻¹ in bioreactors and 0.7 to 1.4 g l⁻¹ in flasks.

**Chemical analyses.** Samples (1 to 60 ml, depending on the culture scale and density of biomass) were removed at intervals and mycelium was separated from the supernatant by filtration through cloth. For analysis of intracellular L-galactonate concentrations, biomass which had been washed first with an equal volume 9 g NaCl l⁻¹, then with distilled water, was frozen at -20°C and subjected to freeze-drying. After weighing, L-galactonate in the dried biomass was extracted in 5 mM H₂SO₄, as described previously for extraction of intracellular 2-keto-3-deoxy-L-galactonate (20). Intracellular amounts are given as mg per g dry biomass, but concentration may be estimated by assuming the volume (ml) of cytoplasm per g dry biomass would be similar to that of *Penicillium chrysogenum*, which has been determined to be 2.86 ml per g dry biomass (15).

The concentration of D-xylose, D-galacturonate and L-galactonate was determined by HPLC using a Fast Acid Analysis Column (100 mm x 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87H organic acid analysis column (300 mm x 7.8 mm, BioRad Laboratories) with 2.5 or 5.0 mM H₂SO₄ as eluant and a flow rate of 0.5 ml min⁻¹. The column was maintained at 55°C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector.
Expression analysis. Samples (1 ml) were collected from flask cultures and the mycelium was harvested by vacuum filtration. The filtered mycelium was immediately frozen with liquid nitrogen and stored at -80°C. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and 1 µg of total RNA was used for cDNA synthesis with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), following the manufacturer’s instructions. cDNA samples were diluted 1:10 with RNase free water (Roche) and 5 µl of diluted cDNA was used for quantitative PCR (qPCR) using a LightCycler II with the LightCycler SYBR green I Master mix (both Roche). The expression of gaaA, An03g01620, An07g00780, An14g04280 and actin were quantified using corresponding primers listed in (Table 1). The level of expression of gaaA and the genes encoding the putative transporters was normalized to actin using the accompanying software (Advance Relative Quantification tool).

Results

Conversion of D-galacturonate to L-galactonate by T. reesei and A. niger (at pH 5.5). Deletion of T. reesei lgd1 (9) and A. niger gaaB resulted in drastically reduced growth of the corresponding strains on D-galacturonic acid when this was provided as the sole carbon source (data not shown). Preliminary experiments demonstrated that both T. reesei Δlgd1 (1.8 g l⁻¹) and A. niger ΔgaaB (5.9 ± 0.1 g l⁻¹) produced L-galactonate when incubated for 120 h in flasks initially containing 10 g l⁻¹ D-galacturonate and 2 g l⁻¹ D-xylose as co-substrate (initial pH 5.1). Less L-galactonate (2.0 ± 0.1 g l⁻¹) was produced by A. niger ΔgaaB when no D-xylose was provided and D-xylose was included as co-substrate in all further experiments.

When cultivated in a bioreactor, T. reesei Δlgd1 L-galactonate production and D-galacturonate utilisation increased with the provision of increasing concentrations of D-xylose as co-substrate (Fig. 2). Up to 7.2 g L-galactonate l⁻¹ were produced in the culture provided 11 g D-
The initial production rate was 0.07 to 0.12 g L-galactonate l$^{-1}$ h$^{-1}$ and final yields were 0.60 to 0.85 g L-galactonate per g D-galacturonate consumed (Fig. 2). Although initial yields of 0.9 to 1.0 g L-galactonate per g D-galacturonate were observed, the yield decreased during the production phase. The biomass concentration also increased with increasing provision of D-xylose (yield 0.5 g biomass [g D-xylose]$^{-1}$), and the specific L-galactonate production rate was lower when 11 g D-xylose l$^{-1}$ was provided than with 3 g l$^{-1}$ (Fig. 2F).

Extracellular L-galactonate was not observed in $T$. reesei $\Delta$lgd1 until D-xylose had been consumed, but L-galactonate was present intracellularly prior to this (Fig. 2). During the production phase there was 40 to 70 mg intracellular L-galactonate [g biomass]$^{-1}$. Intracellular D-galacturonate remained below 2 mg [g biomass]$^{-1}$ (data not shown).

$A$. niger $\Delta$gaaB produced only 1.4 - 1.9 g L-galactonate l$^{-1}$ when cultivated in bioreactors at pH 5.5 (Fig. 2), although 5.9 g l$^{-1}$ had been produced in the preliminary flask experiment. Biomass concentrations were similar to those of $T$. reesei $\Delta$lgd1 (yield 0.56 g biomass [g D-xylose]$^{-1}$), as were intracellular concentrations of L-galactonate (Fig. 2). D-Galacturonate (10-30 mg [g biomass]$^{-1}$) was also detectable in mycelia from the cultures which received 6 or 11 g D-xylose l$^{-1}$. An initial assessment indicated that $gaaA$ expression in this strain was low (data not shown).

**Production of L-galactonate by $A$. niger is sensitive to culture pH.** The modified Vogel's medium used here is not well buffered and thus pH in flask cultures decreased as ammonium was consumed and increased when D-galacturonate was taken up from the medium without release of L-galactonate from the hyphae. Data from the preliminary $A$. niger flask cultures indicated that the highest L-galactonate production rates were observed when pH was low (Fig. 3), and suggested that pH 5.5 may be too high for L-galactonate production by $A$. 
niger. Indeed, L-galactonate production decreased with increasing pH above 5.0 in flask cultures, but was generally high (5-6 g l\(^{-1}\)) at pH values below 5 (Fig. 3). L-Galactonate production was further improved at pH 3 to 4 by cultivating the strain in buffered medium with 20 g D-galacturonate l\(^{-1}\) and 5 g D-xylose l\(^{-1}\) (Fig. 3).

When A. niger ΔgaaB was grown in a pH controlled bioreactor at pH 4.8 with 10 g D-galacturonate l\(^{-1}\) and 6 to 7 g D-xylose l\(^{-1}\), 2.7 g L-galactonate l\(^{-1}\) were produced within 72 h at a rate of 0.04 g l\(^{-1}\) h\(^{-1}\) (yield 0.7 g L-galactonate [g D-galacturonate consumed]\(^{-1}\), Fig. 4). An additional pulse of 8 g D-xylose l\(^{-1}\) was added after 127 h to compensate for the decreasing biomass and a further 2.5 g L-galactonate were produced at the same rate to give a final concentration of 5.4 g l\(^{-1}\) (yield 0.9 g [D-galacturonate consumed]\(^{-1}\), Fig. 4) when the culture was harvested at 171 h. Intracellular L-galactonate accumulation (56 ± 2 mg [g biomass]), Fig. 4) was similar to that observed at pH 5.5 (Fig. 2), but decreased after the addition of D-xylose. D-Galacturonate (<1.6 mg [g biomass]\(^{-1}\)) did not accumulate in the mycelia (data not shown).

**Bioconversion of polygalacturonate to L-galactonate.** A. niger ΔgaaB converted polygalacturonate to L-galactonate at a similar rate (initial rate 0.04 g l\(^{-1}\) h\(^{-1}\), increasing to 0.07 g l\(^{-1}\) h\(^{-1}\) after addition of extra D-xylose) and titre (2.5 g L-galactonate l\(^{-1}\) within 72 h) as it converted the monomer D-galacturonic acid (Fig. 4). L-Galactonate (1.2 g l\(^{-1}\)) was present in the culture supernatant after 26 h, but did not accumulate above 2.8 g l\(^{-1}\) at any time during the cultivation. Addition of D-xylose after 127 h resulted in a total of 6.5 g L-galactonate l\(^{-1}\) (yield 0.85 g L-galactonate [g D-galacturonate consumed]\(^{-1}\)) after 171 h, increasing to 7.6 g l\(^{-1}\) after 195 h. The intracellular concentration of L-galactonate (52 ± 4 mg [g biomass]\(^{-1}\)) was similar to that observed in other L-galactonate producing cultures and also decreased after the addition of D-
xylose (Fig. 4). Low concentrations of D-galacturonate (0.2 to 4.3 mg [g biomass]⁻¹) were also extracted from mycelia incubated in polygalacturonate (data not shown).

**Overexpression of *A. niger gaaA***. Since *gaaA* expression appeared low in the Δ*gaaB* strain, the galacturonate reductase coding gene, *gaaA*, was overexpressed in *A. niger ΔgaaB*. *A. niger* ATTC1015, Δ*gaaB* and the overexpression strain (Δ*gaaB*-gaaA) were grown in modified Vogel's medium with 10 g D-galacturonate l⁻¹ and 2 g D-xylose l⁻¹ at initial pH 3 in flasks. Expression of *gaaA* in *A. niger ΔgaaB* was considerably lower compared to the wild type after 6 h (Table 3). In contrast, in *A. niger ΔgaaB*-gaaA expression of *gaaA* was much higher at 0 and 6 h, as expected (Table 3). After 24 h, *gaaA* expression in *A. niger ΔgaaB* and *A. niger ΔgaaB*-gaaA was similar, whereas its expression in the wild type had decreased (Table 3), probably due to D-galacturonate depletion.

Approximate L-galactonate production rates were determined for the flask cultures. During the first 24 h after inoculation, *A. niger ΔgaaB*-gaaA produced L-galactonate at a significantly (p < 0.05) higher rate (0.070 g L-galactonate l⁻¹ h⁻¹) than *A. niger ΔgaaB* (0.048 g L-galactonate l⁻¹ h⁻¹, Table 4). After 24 h the difference in the production rates of the Δ*gaaB* and Δ*gaaB*-gaaA strains decreased, and after 48 h, when L-galactonate production by both strains was decreasing, their production rates were similar (p > 0.05, 0.046 and 0.054 g L-galactonate l⁻¹ h⁻¹, respectively, Table 4).

The final L-galactonate titres of Δ*gaaB* and Δ*gaaB*-gaaA strains were compared in both modified Vogel's and *A. nidulans* minimal medium in flasks (Table 2). Both L-galactonate titre and yield were generally higher for *A. niger ΔgaaB*-gaaA than for *A. niger ΔgaaB* when grown at pH 3 or 4 in either medium (Table 2). At pH 5 in *A. nidulans* minimal medium, the final L-galactonate titre was notably lower than at pH 4 for both strains and there was no difference
between the strains. However, the yield of L-galactonate on D-galacturonate for *A. niger ΔgaaB*-gaaA was higher than for *A. niger ΔgaaB* also at pH 5 (Table 2).

**Transcription of putative transporter genes in *A. niger ΔgaaB***. The relative transcript levels of 3 genes which have been identified as possible transporters of D-galacturonate (An07g00780, An14g04280, and An03g01620, (12)) were assessed in *A. niger* ATCC1015 and *A. niger ΔgaaB* 3, 6 and 24 h after transfer to D-galacturonic acid containing medium at pH 3 (Table 5). Both An14g04280 and An03g01620 were strongly induced in ATC1015 within 3 hours of the transfer, whereas induction of An07g00780 was only seen 24 h after the transfer. In contrast, no induction of An14g04280 was observed in *A. niger ΔgaaB*. Transcription of An03g1620 and An07g00780 in *A. niger ΔgaaB* was similar to that observed in the control strain.

**Discussion**

Deletion of the gene for the L-galactonate dehydratase, *lgd1* in *T. reesei* or *gaaB* in *A. niger*, resulted in strains that converted D-galacturonate to L-galactonate, which was secreted to the culture supernatant (Figs. 2 to 5). This confirmed that D-galacturonate was still taken up in the deletion strains, as was also the case when either the D-galacturonate reductase (*gar1* or *gaaA* in *T. reesei* and *A. niger*, respectively, (14)) or the 2-keto-3-deoxy-L-galactonate aldolase, *lga1* or *gaaC*, (20) were deleted. In *T. reesei*, the conversion of D-galacturonate to L-galactonate occurred at similar rates (0.07 to 0.12 g L-galactonate l\(^{-1}\) h\(^{-1}\)) as previously reported for the conversion to keto-deoxy-L-galactonate (0.10 to 0.14 g l\(^{-1}\) h\(^{-1}\), (20)), but was faster than the conversion to galactarate (0.024 to 0.046 g l\(^{-1}\) h\(^{-1}\), (14)). In *A. niger*, on the other hand, the conversion of D-galacturonate to L-galactonate (0.04 to 0.07 g L-galactonate l\(^{-1}\) h\(^{-1}\)) was much
slower than the conversion to keto-deoxy-L-galactonate (0.27 to 0.33 g l⁻¹ h⁻¹, (20)), suggesting
that the disruption of the pathway at the earlier step created additional constraints in this strain.

The yield of L-galactonate from D-galacturonate was 0.6 to 0.8 g g⁻¹ for T. reesei Δlgd1
and 0.7 to 0.9 for A. niger ΔgaaB. Thus, the yields were only slightly lower than the theoretical
yield (1.0 g L-galactonate [g D-galacturonate]⁻¹), but still indicated that some of either the D-
galacturonate or the produced L-galactonate were consumed in unidentified metabolic
reaction(s). Futile consumption of D-galacturonate has been observed previously in strains
deleted of gaaA/gar1 or gaaC/lga1 (14, 20), but the fate of the carbon remains unclear since
there is no measureable production of biomass from D-galacturonate in these strains.

Although production of both L-galactonate and keto-deoxy-L-galactonate require NADPH
as a co-factor for the D-galacturonate reductase, L-galactonate production was more dependent
on the addition of D-xylose as a co-substrate (Fig. 2) to obtain good production than was the
production of the keto-deoxy derivative. This may reflect a greater need for energy in the export
of L-galactonate, since we observed that the intracellular concentration of L-galactonate (40 to
70 mg L-galactonate [g biomass]⁻¹ in both T. reesei and A. niger) was higher than the maximum
intracellular concentrations of keto-deoxy-L-galactonate (35 to 45 mg L-galactonate [g biomass]⁻¹
in the corresponding strains (20). After provision of additional co-substrate to A. niger ΔgaaB
cultures at pH 4.5 to 4.8 the intracellular L-galactonate concentration decreased to around 23 mg
[g biomass]⁻¹ (Fig. 4), supporting the hypothesis that energy is needed for export.

Assuming the volume of cytoplasm to be approximately 2.86 times the dry biomass (10),
the average intracellular concentration of L-galactonate was ~20 g l⁻¹ and was much higher than
the L-galactonate concentration in the medium. This also suggests that export may be a
bottleneck in extracellular production. In addition, the high intracellular concentration of L-
galactonate may limit the rate of D-galacturonate conversion by feedback inhibition and/or providing substrate for the reverse reaction, which has been shown to occur with both the \textit{T. reesei} gar1 (10) and the \textit{A. niger} gaaA (13) D-galacturonate reductases. The $K_m$ for L-galactonate of \textit{T. reesei} gar1 is 4 mM (0.8 g l$^{-1}$) (10), which is much lower than the intracellular L-galactonate concentrations observed. Thus, accumulation of L-galactonate may limit the reaction more than accumulation of keto-deoxy-L-galactonate, since the action of the L-galactonate dehydratase is irreversible (9). Generation of intracellular D-galacturonate may also have affected uptake of the substrate, about which little is known in filamentous fungi. Intracellular D-galacturonate was, however, only observed in \textit{A. niger} and not in \textit{T. reesei}.

In contrast to keto-deoxy-L-galactonate production (20), L-galactonate production was more efficient in \textit{T. reesei} than in \textit{A. niger} at pH 5.5, producing higher titres at higher rates (Fig. 2). \textit{T. reesei} was also found to be more effective than \textit{A. niger} in the production of galactarate (14), and these results confirm that \textit{T. reesei} is an interesting and useful host for organic acid production, even though it is not known as a high producer of organic acids, nor tolerant to very low culture pH.

Low galactarate production by \textit{A. niger} $\Delta$gaaA-udh was attributed to subsequent metabolism of the galactarate (14). Metabolism of L-galactonate appeared negligible (Fig. 4) or limited (Fig. 2) in \textit{A. niger} $\Delta$gaaB, rather L-galactonate production by \textit{A. niger} was found to be pH-dependent, with the highest production rates and titres observed at pH values below 5.0 and no reduction in production even at pH 3.0 (Fig. 3). At pH 4.5 to 4.8, production of L-galactonate by \textit{A. niger} $\Delta$gaaB was as good as that of \textit{T. reesei} $\Delta$lgd1 at pH 5.5. At low extracellular pH, more of the product is protonated to L-galactonic acid ($pK_a$ ~3.5) creating a greater difference in concentration between the dissociated intra- and extracellular L-galactonate pools. If the
protonated organic acid is not re-imported to the cytoplasm, then a low extracellular pH can provide the dominant driving force for organic acid export, as has been predicted for citrate export from *A. niger* (2). Further, low extracellular pH may influence the transport of D-galacturonic acid (pKa=3.51). However, *A. niger* transported D-galacturonate at much higher rates when producing keto-deoxy-L-galactonate at pH 5.5 (0.12 to 0.56 g l\(^{-1}\) h\(^{-1}\), (20)) or galactarate at pH 5.0 (0.21 to 0.46 g l\(^{-1}\) h\(^{-1}\), (14)) than observed during L-galactonate production at any pH (0.04 to 0.15 g l\(^{-1}\) h\(^{-1}\), Fig. 2 and data not shown). Thus improved uptake at low pH is unlikely to explain the improved L-galactonate production observed.

D-galacturonate is an inducer of the D-galacturonate pathway genes *gaaA*, *gaaB* and *gaaC* in *A. niger* ATCC1015, CBS120.49 and Δ*gaaA* strain (12, 14). In ATCC1015, the transcription of these three genes was induced simultaneously within 4 hours of transfer to D-galacturonate and induction of *gaaB* and *gaaC* remained similar in *A. niger* Δ*gaaA* compared to ATCC1015 (14). In this study, we observed that *gaaA* was not induced in *A. niger* Δ*gaaB* even 6 hours after exposure to D-galacturonate (Table 3), although transcription had increased after 24 hours. In ATCC1015, *gaaA* expression was already reduced after 24 h incubation, due to D-galacturonate depletion. Induction of the gene encoding the third enzyme of the pathway, *gaaC*, was similarly delayed in *A. niger* Δ*gaaB* (J. Kuivanen, unpublished data), suggesting that the induction of the entire pathway was affected by the deletion of *gaaB*. The similar transcriptional response of *gaaA* and *gaaC* might be expected since these genes share a common bidirectional promoter (13). The altered transcription profiles of the genes in the Δ*gaaB* strain suggest that L-galactonate, keto-deoxy-L-galactonate or L-galactonate dehydratase itself may have roles in transcriptional regulation of the D-galacturonate pathway genes. Regardless of the regulatory
mechanism, the delayed induction of *gaaA* in the Δ*gaaB* strain would account for low initial
rates of D-galacturonate conversion.

In order to eliminate *gaaA* induction as a rate limiting factor for L-galactonate production, *gaaA* was overexpressed under the *gpdA* promoter in *A. niger ΔgaaB*. The L-galactonate production rate was initially significantly (p<0.002) higher in *A. niger ΔgaaB-gaaA* compared to *A. niger ΔgaaB* in flasks at pH 3 (Table 4), indicating that low *gaaA* expression was indeed a rate limiting factor. However, *gaaA* was expressed under the *gpdA* promoter, which gives less induction in the absence of a metabolisable carbon source (here D-xylose), even though it is generally described as constitutive. Thus, expression of *gaaA* decreased during the expression studies. After 24 h, when *gaaA* expression had been induced in the *gaaB* deletion strain, the production rates of *A. niger ΔgaaB-gaaA* and *A. niger ΔgaaB* were similar (Table 4). The initial improvement in production resulted in 24 to 39% more L-galactonate being produced at pH 3 when *gaaA* was overexpressed than when it was not, with corresponding improvements in the conversion efficiency and yield (Table 2). Interestingly, the benefit of overexpression of *gaaA* was pH dependent even though *gaaA* expression was not (J. Kuivinen, unpublished data), with the greatest benefit at pH 3, although smaller improvements in yield were also observed at higher pH values (Table 2).

D-Xylose was previously found to be a good co-substrate in the production of keto-deoxy-L-galactonate (20), but D-Galacturonate did not appear to be taken up while D-xylose was being consumed (Fig. 2). Limited D-galacturonate uptake during the time when *gaaA* expression was high in *A. niger ΔgaaB-gaaA* probably limited the improvement in L-galactonate production which could be achieved by this strain. In addition, only two of the three putative D-galacturonate transporters (12) were induced in the Δ*gaaB* strain (Table 5). The roles of these
putative transporters is not known, but the limited D-galacturonate transport in *A. niger ΔgaaB*
and *A. niger ΔgaaB-gaaA* may indicate that the protein encoded by An14g04280 has a dominant
role.

Despite the fact that production of L-galactonate with *A. niger ΔgaaB* required more
investigation and additional strain development than with *T. reesei Δlgd1*, *A. niger* is more
suitable for development of a consolidated L-galactonate production process, which would use
less processed polymeric substrates, such as polygalacturonate, pectin, or even raw, untreated
biomass. *A. niger* produces a more complex spectrum of pectinases than *T. reesei*, which is
unable to degrade pectin (20). Using the current *A. niger ΔgaaB* strain, production of L-
galactonate from polygalacturonate was found to be as efficient as production from the D-
galacturonate monomer (Fig. 4). Thus, a high concentration of extracellular D-galacturonate was
not necessary to sustain its uptake and the slow release of monomer may be beneficial in
providing continual induction of the native *gaaA* gene. Polygalacturonate was used as a substrate
here, but these results suggest that L-galactonate could also be produced directly from pectin,
which would require less processing and would also provide the co-substrates (e.g. D-galactose,
D-xylose, L-arabinose) for the initial production of biomass and NADPH. A more gradual
provision of co-substrate in a fed-batch or continuous process may also be useful, since this
would ensure that production rates did not decrease as a result of cell lysis after the co-substrate
was consumed and for the ΔgaaB-gaaA strain would sustain higher expression levels of *gaaA*.

D-Galactonate has been produced in high concentration from D-galactose using
*Gluconobacter oxydans* (18), but this is the first report of extracellular production of L-
galactonate in gram quantities from D-galacturonic and polygalacturonic acids. Its production
has led to further insights into D-galacturonate metabolism in *A. niger*, while further
enhancement in production by both strain engineering and process development may provide an efficient source of L-galactonate for e.g. microbial ascorbic acid production and other applications.

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References


TABLE 1 Primers used to generate vectors for deletion of *gaaB* and incorporation of *gaaA* in *A. niger* ATCC1015 Δ*pyrG*, to confirm integration and for qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gaaB-5-F</td>
<td>TATACTCGAGAGTTCCCTCGATCAGGAACGA</td>
</tr>
<tr>
<td>gaaB-5-R</td>
<td>TATAGAGCTCGCAATCTAGTGGCAATGC</td>
</tr>
<tr>
<td>gaaB-3-F</td>
<td>TATAGAGCTCGCATTACATTGGTTATGTTGGG</td>
</tr>
<tr>
<td>gaaB-3-R</td>
<td>TATAGAATTACAGATATTGCACCGAGAA</td>
</tr>
<tr>
<td>pyrG-del-F_n</td>
<td>TATACCCGGGTGATTGAGGTGATTGGCGAT</td>
</tr>
<tr>
<td>pyrG-del-R_n</td>
<td>TATACCCGGTTATACCGACGACGACAT</td>
</tr>
<tr>
<td>gaaB-ORF-F</td>
<td>AGATACAAGTTTCACCACGA</td>
</tr>
<tr>
<td>gaaB-ORF-R</td>
<td>GCCCCTCCAGAAATGGTCTT</td>
</tr>
<tr>
<td>gaaA-exp-F</td>
<td>ATGAATTCGAGCTCCACAATGGCTCCCCCAG</td>
</tr>
<tr>
<td>gaaA-exp-R</td>
<td>AGGCACGCTCCGGCTACTTCAGCTCCACCTTTC</td>
</tr>
<tr>
<td>gpdA-F</td>
<td>AAGTGGAAGGCTGGGTGC</td>
</tr>
<tr>
<td>gaaA_qPCR_F</td>
<td>AGGACACGATTACTCTACTTGTG</td>
</tr>
<tr>
<td>gaaA_qPCR_R</td>
<td>GAGCCCATATAATGGGAAGTACTG</td>
</tr>
<tr>
<td>act_qPCR_F</td>
<td>CAACATTGTGCTATGGCTGGGT</td>
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<tr>
<td>act_qPCR_R</td>
<td>GGAGGAGCAATGATCTTGAAC</td>
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<td>An07g00780_qPCR_F</td>
<td>CTATCATCAATGGCCTCCC</td>
</tr>
<tr>
<td>An07g00780_qPCR_R</td>
<td>CCACTGACGAAGCCATAGAC</td>
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<tr>
<td>An14g04280_qPCR_F</td>
<td>GTATGTGAGCGAGATCTTCCC</td>
</tr>
<tr>
<td>An14g04280_qPCR_R</td>
<td>TTTCCCTGGGCGAAGACAATGAC</td>
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<tr>
<td>An03g01620_qPCR_F</td>
<td>GGAATACGAAGAAGTGCAGGA</td>
</tr>
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<td>----------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>An03g01620_qPCR_R</td>
<td>GGTGTTTCCAGACATGCCAG</td>
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TABLE 2 L-Galactonate (L-GalA) production at 144 h by *A. niger ΔgaaB* and the ΔgaaB strain
overexpressing *gaaA* (ΔgaaB-gaaA) in buffered *A. nidulans* minimal medium (MM) with 20 g
D-galacturonate l⁻¹ and 5 g D-xylose l⁻¹ and in modified Vogel’s medium with 10 g D-
galacturonate l⁻¹ and 2 g D-xylose l⁻¹ in flasks at initial pH 3, 4 or 5. Mean ± SEM (n = 3). The
conversion and yield on D-galacturonic acid (D-GalUA) are also shown.

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Strain</th>
<th>L-GalA (g l⁻¹)</th>
<th>Conversion (g g⁻¹)</th>
<th>Yield (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em> MM</td>
<td>5</td>
<td>ΔgaaB</td>
<td>4.1 ± 0.2</td>
<td>0.20</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔgaaB-gaaA</td>
<td>4.1 ± 0.3</td>
<td>0.20</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ΔgaaB</td>
<td>7.2 ± 0.8</td>
<td>0.35</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔgaaB-gaaA</td>
<td>7.8 ± 0.4</td>
<td>0.38</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ΔgaaB</td>
<td>6.3 ± 0.1</td>
<td>0.31</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔgaaB-gaaA</td>
<td>8.7 ± 0.2</td>
<td>0.43</td>
<td>1.00</td>
</tr>
<tr>
<td>modified Vogel's</td>
<td>4</td>
<td>ΔgaaB</td>
<td>4.2 ± 0.1</td>
<td>0.41</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔgaaB-gaaA</td>
<td>5.0 ± 0.1</td>
<td>0.49</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>ΔgaaB</td>
<td>4.9 ± 0.1</td>
<td>0.47</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ΔgaaB-gaaA</td>
<td>6.2 ± 0.3</td>
<td>0.59</td>
<td>0.82</td>
</tr>
</tbody>
</table>
**TABLE 3** Relative expression of *gaaA* in *A. niger* ATCC1015, Δ*gaaB* and Δ*gaaB-gaaA* when grown in flasks in modified Vogel’s medium with 10 g D-galacturonate l⁻¹ and 2 g D-xylose l⁻¹ at initial pH 3.0. Average ± SEM (n = 3).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ATCC1015</th>
<th>Δ<em>gaaB</em></th>
<th>Δ<em>gaaB-gaaA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>14.0 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>3.6 ± 0.6</td>
<td>0.1 ± 0.0</td>
<td>16.6 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>2.6 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>9.5 ± 0.6</td>
</tr>
<tr>
<td>24</td>
<td>0.2 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>
TABLE 4 L-Galactonate production rates for *A. niger ΔgaaB* and ΔgaaB-gaaA when grown in flasks in modified Vogel’s medium with 10 g D-galacturonate l⁻¹ and 2 g D-xylose l⁻¹ at initial pH 3.0. Values (average ± SEM, n = 3) in the same row with different superscripts a or b differed significantly (p < 0.05).

<table>
<thead>
<tr>
<th>Time interval (h)</th>
<th>ΔgaaB</th>
<th>ΔgaaB-gaaA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td>0.048 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.070 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>24-48</td>
<td>0.064 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>48-78</td>
<td>0.046 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.054 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
TABLE 5 Relative expression of putative transporters An07g00780, An14g04280, and An03g01620 in *A. niger* ATCC1015 and ΔgaaB when grown in flasks in modified Vogel’s medium with 10 g D-galacturonate l\(^{-1}\) and 2 g D-xylose l\(^{-1}\) at initial pH 3.0. Average ± SEM (n = 3), n.d. = no data.

<table>
<thead>
<tr>
<th>Putative transporter</th>
<th>Time (h)</th>
<th>ATCC1015</th>
<th>ΔgaaB</th>
</tr>
</thead>
<tbody>
<tr>
<td>An07g00780</td>
<td>0</td>
<td>0.4 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.1 ± 0.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.1 ± 0.3</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>An14g04280</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.1 ± 0.2</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.9 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>An03g01620</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.0 ± 0.5</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>
Figure legends

FIG 1 The fungal D-galacturonic acid pathway. The genes encoding the enzymes in *T. reesei* and *A. niger* are indicated. The deletion of *lgd1* in *T. reesei* and *gaaB* in *A. niger* disrupted the pathway and generated strains which accumulated L-galactonate.

FIG 2 Concentrations of extracellular A) L-galactonate, B) biomass, C) D-galacturonate, D) D-xylose, and intracellular E) L-galactonate from *T. reesei* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) in modified Vogel's medium initially containing 10 g D-galacturonate l⁻¹, and 2.5 or 3, 6 or 11 g D-xylose l⁻¹, as indicated, at pH 5.5, 800 rpm, 1.6vvm aeration, 30°C. F) The effect of D-xylose concentration on the yield of L-galactonate on D-galacturonate consumed, and the volumetric production and specific production rates of L-galactonate for *T. reesei* Δ*lgd1*. Error bars represent ± SEM (n = 2).

FIG 3 L-Galactonate production by *A. niger* Δ*gaaB* in flasks. A) L-Galactonate production rate as a function of pH for unbuffered cultures provided 10 g D-galacturonate l⁻¹ at initial pH 5.2, with no (open symbols) or 2 g l⁻¹ D-xylose (solid symbols) provided for growth. Error bars represent ± SEM, n = 3. B) Concentration of L-galactonate produced in 120-144 h in unbuffered modified Vogel's medium containing 10 g D-galacturonate l⁻¹ and 2 g D-xylose l⁻¹ (solid symbols) and in buffered *A. nidulans* medium containing 20 g D-galacturonate l⁻¹ and 5 g D-xylose l⁻¹ (open symbols). The pH of the media was initially adjusted to 3, 4, 5 or 6, but average culture pH is shown. Error bars represent ± SEM for 3 to 6 replicate cultures and where not visible are smaller than the symbol.

FIG 4 Concentration of L-galactonate, biomass and intracellular L-galactonate in cultures of *A. niger* Δ*gaaB* in modified Vogel's medium with 5 g D-xylose l⁻¹ and containing 10 g D-
galacturonate l⁻¹ (open symbols, pH 4.8) or 15 g polygalacturonate l⁻¹ (solid symbols pH 4.5).

Cultures were maintained at 30°C, 800 rpm, 1.6 vvm aeration and were given an additional 9 g D-xylose l⁻¹ at 127.8 h. Error bars represent ± SEM (n = 2) and where not visible are smaller than the symbol.