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Engineering Filamentous Fungi for Conversion of D-Galacturonic Acid to L-Galactonic Acid

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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 hydrolyzed. 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* Δ*lgd1* and *Aspergillus niger* Δ*gaaB* strains produced L-galactonate at yields of 0.6 to 0.9 g per g of substrate consumed. Although *T. reesei* Δ*lgd1* could produce L-galactonate at pH 5.5, a lower pH was necessary for *A. niger* Δ*gaaB*. Provision of a cosubstrate improved the production rate and titer 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* Δ*gaaB* produced L-galactonate from polygalacturonate as efficiently as from the monomer.

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 $\times$ 10^6 and 115 $\times$ 10^6 metric tons, respectively. After beet processing, 5 to 10% of the sugar beet remains as dried sugar beet pulp. This pulp contains ca. 25% pectin (6). Citrus peel contains ca. 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 the pulp and peel are dried since; otherwise, they rot rapidly. Disposal of the material is problematic because of the bad odor 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 (6). This process is only economical when done on a 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 flavor 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 (5, 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 valorize 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 catabolized through a pathway (Fig. 1) that includes reactions catalyzed 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 metabolized 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 gene, *lgd1*, was deleted was unable to grow on D-galacturonic acid (9). In the present study 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

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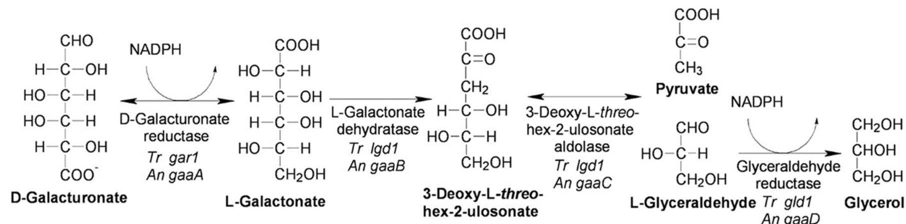

FIG 1 Fungal D-galacturonic acid pathway. The genes encoding the enzymes in *T. reesei* (*Tr*) and *A. niger* (*An*) are indicated. The deletion of *lgd1* in *T. reesei* and *gaaB* in *A. niger* disrupted the pathway and generated strains that accumulated L-galactonate.

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 physicochemical 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, and 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 that forms from L-galactonic acid at acidic pH can be oxidized to L-ascorbic acid chemically (3) or in a fermentative process (17).

MATERIALS AND METHODS

Strains. The deletion of the *lgd1* in *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) was described previously (9). *Aspergillus niger* ATCC 1015 (Δ*pyrG*), with the gene encoding the orotidine-5′-phosphate decarboxylase (i.e., the *pyrG* gene) deleted (14), was used to construct the *gaaB* deletion strain. The cassette for deletion of *gaaB* contained 1,550 bp from the *A. niger gaaB* promoter, 1,533 bp from the *A. niger gaaB* terminator, and a 1,920-bp fragment containing the *pyrG* gene flanked with its native promoter and terminator. These fragments were obtained by PCR of *A. niger* ATCC 1015 genomic DNA using the primers gaaB-5-F, gaaB-5-R, gaaB-3-F, gaaB-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 1), and the proofreading DNA polymerase Phusion (Finnzymes). Plasmid pRSET-A (Invitrogen) was digested with EcoRI and PvuII (both NEB) and the terminator fragment (*gaaB-3*) with EcoRI to produce an intermediary construct by ligation using T4 DNA ligase (NEB). This intermediary construct was digested with XhoI (NEB) and Ecl136II (Fermentas) and ligated to the XhoI-digested promoter fragment (*gaaB-5*). The resulting vector was digested with Ecl136II and treated with phosphatase. The *pyrG* DNA fragment, after digestion with SmaI, was inserted between the two *gaaB* flanking regions. The deletion cassette, 5,006 bp containing the *gaaB* flanking regions and the *pyrG* gene, was released by EcoRI+XhoI digestion and introduced into the *A. niger* ATCC 1015 Δ*pyrG* strain 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.

TABLE 1 Primers used to generate vectors for the deletion of *gaaB* and the incorporation of *gaaA* in *A. niger* ATCC 1015 Δ*pyrG* in order to confirm integration and for quantitative PCR

Primer	Sequence (5′–3′)
gaaB-5-F	TATACTCGAGAGTTCCTCGATCAGGAACGA
gaaB-5-R	TATAGAGCTCGCAATCTAGTTGCAATGC
gaaB-3-F	TATAGAGCTCGCATTACATTGGTTATGTGGG
gaaB-3-R	TATAGAATTCAGACATTAGTCCCCGAGAA
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
gaaB-ORF-F	AGATCACAAGTTTCACCACGA
gaaB-ORF-R	GCCCCTCCAGAATGGTCTT
gaaA-exp-F	ATGAATTCGAGCTCCACAATGGCTCCCCCAG
gaaA-exp-R	AGGCGCGCCCGGGCTACTTCAGCTCCCACTTTC
gpdA-F	AAGTGGAAAGGCTGGTGTGC
gaaA_qPCR_F	AGGACACGATTACTCTACTTGTG
gaaA_qPCR_R	GAGCCCATATAATGGAAGTACTG
act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC
An07g00780_qPCR_F	CTATCATCAATGCCGCCTCC
An07g00780_qPCR_R	CCACTGACGAAGCCATAGAC
An14g04280_qPCR_F	GTATGTGAGCGAGATCTTCCC
An14g04280_qPCR_R	TTTCCTTGGCGAAGACAATGAC
An03g01620_qPCR_F	GGAATACGAAGAAGTGCAGGA
An03g01620_qPCR_R	GGTGTTTCCAGACATGCCAG

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 ATCC 1015 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 the *A. niger* ATCC 1015 *gaaB*Δ strain by transformation. Transformants were screened for integration of the *gpdA-gaaA-trpC-hph* cassette by growth in the presence of 400 μg of hygromycin B (Calbiochem) ml^{-1}. 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 $liter^{-1}$) was provided as a carbon source, and ammonium sulfate (1.65 or 3.3 g $liter^{-1}$) was provided as a nitrogen source. D-Galacturonate (~10 g $liter^{-1}$; prepared as sodium salt), or polygalacturonate (15 g $liter^{-1}$; prepared as a sodium salt and containing 11 g of D-galacturonic acid $liter^{-1}$ plus 1 g of combined D-xylose, D-galactose, and D-mannose $liter^{-1}$ when hydrolyzed) 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 of D-galacturonate $liter^{-1}$ and 5 g of D-xylose $liter^{-1}$. The pH of production medium was adjusted between 3.0 and 6.0 with NaOH.

Medium (modified from Vogel [19]) for precultures contained 20 g of D-xylose $liter^{-1}$ and was supplemented with 1 g of Bacto peptone $liter^{-1}$ to provide more rapid growth in this chemically defined medium. *A. niger* precultures also contained 4 g of agar $liter^{-1}$ or 30 g of gelatin $liter^{-1}$, so that growth would be more filamentous. Agar was used in precultures for bioreactor cultures, since it was not metabolized by *A. niger*, and thus the biomass received the same nutrients as the *T. reesei* precultures. For studies of gene expression, precultures of *A. niger* were grown in medium containing 10 g of yeast extract $liter^{-1}$, 20 g of peptone $liter^{-1}$, and 30 g of gelatin $liter^{-1}$.

Cultural conditions. Small-scale cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium and incubated at 30°C and 200 rpm. Preculture flasks were inoculated with conidial suspensions (final concentrations, 5.3×10^5 conidia ml^{-1}), and production flasks were inoculated with mycelium from the precultures. *T. reesei* precultures 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, Finland) and rinsed with sterile H_2O (>2 volumes) to remove residual peptone and D-xylose. *A. niger* was grown for 24 h in preculture medium containing 4 g of agar $liter^{-1}$ or 30 g of gelatin $liter^{-1}$ to reduce formation of pellets. Mycelium (5 ml) from agar-containing precultures was transferred to fresh preculture medium lacking agar (50 ml) and incubated for 18 h to reduce the agar content in the cultures and provide an inoculum consisting of very small (<2-mm-diameter) pellets for D-galacturonate conversion, which could be filtered and washed in the same manner as the *T. reesei* precultures. Alternatively, gelatin-containing precultures were harvested by vacuum filtration and rinsed with sterile H_2O warmed to 37°C to remove gelatin and then with cold H_2O. Washed mycelium was aseptically transferred to production medium.

For larger-scale cultures, mycelium was grown in bioreactors in 500 ml (Multifors; maximum working volume, 500 ml; Infors HT, Switzerland). Cultures were maintained at 30°C and 800 rpm, with a 1.6 volume of gas volume $culture^{-1}$ min^{-1} (vvm). The 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 H_3PO_4. Polypropylene glycol (mixed molecular weight [21]) was added to control foam production. The initial biomass concentration in *T. reesei* cultures was 0.3 g $liter^{-1}$, and in *A. niger* cultures the concentrations were 0.4 g $liter^{-1}$ in bioreactors and 0.7 to 1.4 g $liter^{-1}$ in flasks.

Chemical analyses. Samples (1 to 60 ml, depending on the culture scale and density of biomass) were removed at intervals, and the mycelium was separated from the supernatant by filtration through cloth. For analysis of intracellular L-galactonate concentrations, biomass that had been washed first with an equal volume of 9 g of NaCl $liter^{-1}$ and then with distilled water was frozen at −20°C and subjected to freeze-drying. After the sample was weighed, the L-galactonate in the dried biomass was extracted in 5 mM H_2SO_4 as described previously for the extraction of intracellular 2-keto-3-deoxy-L-galactonate (20). Intracellular amounts are given as mg per g of dry biomass, but the concentration may be estimated by assuming that the volume (in ml) of cytoplasm per g of dry biomass would be similar to that of *Penicillium chrysogenum*, which has been determined to be 2.86 ml per g of dry biomass (15).

The concentrations of D-xylose, D-galacturonate, and L-galactonate were determined by HPLC using a fast acid analysis column (100 by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) linked to an Aminex HPX-87H organic acid analysis column (300 by 7.8 mm; Bio-Rad Laboratories) with 2.5 or 5.0 mM H_2SO_4 as the 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 minikit (Qiagen), and 1 μg of total RNA was used for cDNA synthesis with a Transcriptor high-fidelity cDNA synthesis kit (Roche) according to 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 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 the corresponding primers listed in Table 1. The level of expression of *gaaA* and the genes encoding the putative transporters was normalized to actin by using the accompanying software (Advance Relative Quantification tool).

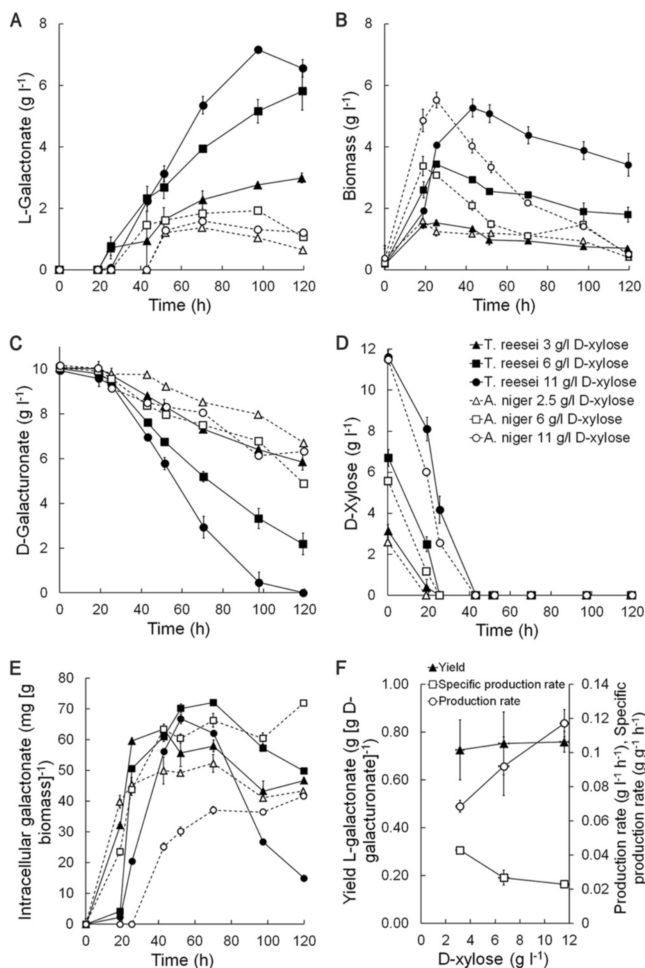

FIG 2 (A to E) Concentrations of extracellular L-galactonate (A), biomass (B), D-galacturonate (C), and D-xylose (D) and intracellular L-galactonate from *T. reesei* Δ*lgd1* (solid symbols) and *A. niger* Δ*gaaB* (open symbols) (E) in modified Vogel medium initially containing 10 g of D-galacturonate $liter^{-1}$ and 2.5, 3, 6, or 11 g of D-xylose $liter^{-1}$, as indicated, at pH 5.5, 800 rpm, 1.6-vvm aeration, and 30°C. (F) Effect of D-xylose concentration on yield of L-galactonate on D-galacturonate consumed, and volumetric production and specific production rates of L-galactonate for *T. reesei* Δ*lgd1*. Error bars represent ± the standard error of the mean (SEM; $n = 2$).

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* Δ*lgd1* (1.8 g $liter^{-1}$) and *A. niger* Δ*gaaB* (5.9 ± 0.1 g $liter^{-1}$) produced L-galactonate when incubated for 120 h in flasks initially containing 10 g of D-galacturonate $liter^{-1}$ and 2 g of D-xylose $liter^{-1}$ as cosubstrates (initial pH of 5.1). Less L-galactonate (2.0 ± 0.1 g $liter^{-1}$) was produced by *A. niger* Δ*gaaB* when no D-xylose was provided, and thus D-xylose was included as a cosubstrate in all further experiments.

When *T. reesei* Δ*lgd1* was cultivated in a bioreactor, L-galactonate production and D-galacturonate utilization increased with the provision of increasing concentrations of D-xylose as cosubstrate (Fig. 2). Up to 7.2 g of L-galactonate $liter^{-1}$ was produced in

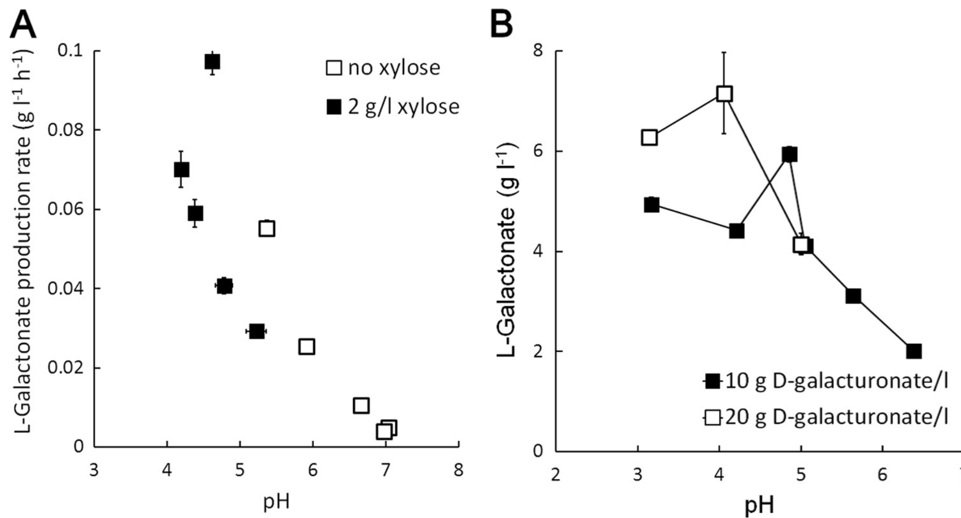

FIG 3 L-Galactonate production by *A. niger* Δ*gaaB* in flasks. (A) L-Galactonate production rate as a function of pH for unbuffered cultures provided 10 g of D-galacturonate liter^{-1} at an initial pH of 5.2 with no D-xylose (open symbols) or 2 g of D-xylose liter^{-1} (solid symbols) provided for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

the culture provided 11 g of D-xylose liter^{-1}. The initial production rate was 0.07 to 0.12 g of L-galactonate liter^{-1} h^{-1}, and the final yields were 0.60 to 0.85 g of L-galactonate per g of D-galacturonate consumed (Fig. 2). Although initial yields of 0.9 to 1.0 g of L-galactonate per g of D-galacturonate were observed, the yield decreased during the production phase. The biomass concentration also increased with increasing provision of D-xylose (yield of 0.5 g biomass g D-xylose^{-1}), and the specific L-galactonate production rate was lower when 11 g of D-xylose liter^{-1} was provided than with 3 g liter^{-1} (Fig. 2F).

Extracellular L-galactonate was not observed in *T. reesei* Δ*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 of intracellular L-galactonate g biomass^{-1}. Intracellular D-galacturonate remained <2 mg g biomass^{-1} (data not shown).

A. niger Δ*gaaB* produced only 1.4 to 1.9 g of L-galactonate liter^{-1} when cultivated in bioreactors at pH 5.5 (Fig. 2), although 5.9 g liter^{-1} had been produced in the preliminary flask experiment. The biomass concentrations were similar to those of *T. reesei* Δ*lgd1* (yield of 0.56 g biomass g D-xylose^{-1}), as were the intracellular concentrations of L-galactonate (Fig. 2). D-Galacturonate (10 to 30 mg g biomass^{-1}) was also detectable in mycelia from the cultures which received 6 or 11 g of D-xylose liter^{-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 medium used here is not well buffered, and thus the 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. The 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 to 6 g liter^{-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 of D-galacturonate liter^{-1} and 5 g of D-xylose liter^{-1} (Fig. 3).

When *A. niger* Δ*gaaB* was grown in a pH-controlled bioreactor at pH 4.8 with 10 g of D-galacturonate liter^{-1} and 6 to 7 g of D-xylose liter^{-1}, 2.7 g of L-galactonate liter^{-1} was produced within 72 h at a rate of 0.04 g liter^{-1} h^{-1} (yield of 0.7 g of L-galactonate per g of D-galacturonate consumed; Fig. 4). An additional pulse of 8 g of D-xylose liter^{-1} was added after 127 h to compensate for the decreasing biomass, and a further 2.5 g of L-galactonate was produced at the same rate to give a final concentration of 5.4 g liter^{-1} (yield of 0.9 g per g of D-galacturonate consumed; Fig. 4) when the culture was harvested at 171 h. Intracellular L-galactonate accumulation (56 ± 2 mg g biomass^{-1}; 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* Δ*gaaB* converted polygalacturonate to L-galactonate at a similar rate (initial rate of 0.04 g liter^{-1} h^{-1}, increasing to 0.07 g liter^{-1} h^{-1} after the addition of extra D-xylose) and titer (2.5 g of L-galactonate liter^{-1} within 72 h) as it converted the monomer D-galacturonate (Fig. 4). L-Galactonate (1.2 g liter^{-1}) was present in the culture supernatant after 26 h but did not accumulate above 2.8 g liter^{-1} at any time during the cultivation. The addition of D-xylose after 127 h resulted in a total of 6.5 g of L-galactonate liter^{-1} (yield of 0.85 g of L-galactonate per g of D-galacturonate consumed^{-1}) after 171 h, increasing to 7.6 g liter^{-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

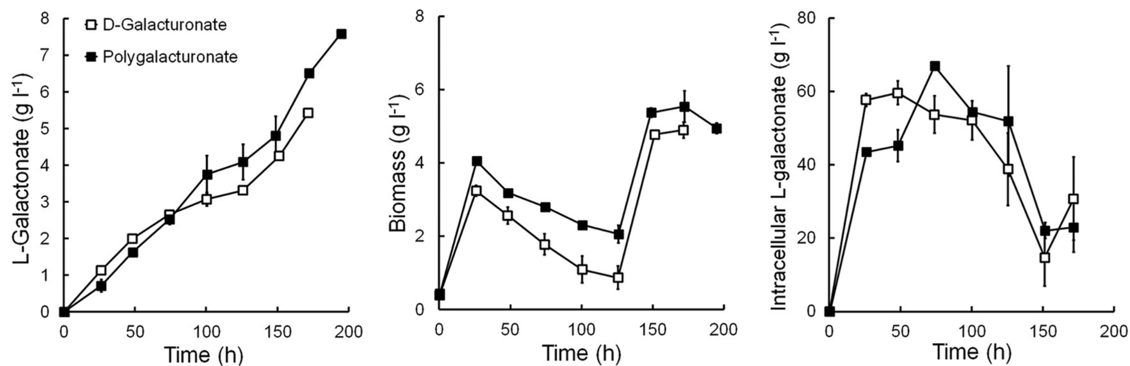

FIG 4 Concentrations of L-galactonate, biomass, and intracellular L-galactonate in *A. niger* Δ*gaaB* cultures in modified Vogel medium with 5 g of D-xylose liter^{-1} and containing 10 g of D-galacturonate liter^{-1} (open symbols, pH 4.8) or 15 g of polygalacturonate liter^{-1} (solid symbols pH 4.5). The cultures were maintained at 30°C, 800 rpm, and 1.6-vvm aeration and were given an additional 9 g of D-xylose liter^{-1} at 127.8 h. Error bars represent ± the SEM ($n = 2$) and, where not visible, are smaller than the symbol.

mg g biomass^{-1}) were also extracted from mycelia incubated in polygalacturonate (data not shown).

Overexpression of *A. niger gaaA*. Since *gaaA* expression appeared to be low in the Δ*gaaB* strain, the galacturonate reductase coding gene, *gaaA*, was overexpressed in *A. niger* Δ*gaaB*. *A. niger* ATCC 1015, the Δ*gaaB* strain, and the overexpression strain (Δ*gaaB-gaaA*) were grown in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3 in flasks. The expression of *gaaA* in *A. niger* Δ*gaaB* was considerably lower compared to the wild type after 6 h (Table 2). In contrast, in *A. niger* Δ*gaaB-gaaA* expression of *gaaA* was much higher at 0 and 6 h, as expected (Table 2). After 24 h, *gaaA* expression levels in *A. niger* Δ*gaaB* and *A. niger* Δ*gaaB-gaaA* were similar, whereas its expression in the wild type had decreased (Table 2), 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* Δ*gaaB-gaaA* produced L-galactonate at a significantly ($P < 0.05$) higher rate (0.070 g of L-galactonate liter^{-1} h^{-1}) than *A. niger* Δ*gaaB* (0.048 g of L-galactonate liter^{-1} h^{-1}; Table 3). 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 of L-galactonate liter^{-1} h^{-1}, respectively; Table 3).

The final L-galactonate titers of the Δ*gaaB* and Δ*gaaB-gaaA* strains were compared in both modified Vogel medium and *A. nidulans* minimal medium in flasks (Table 4). Both the L-galactonate titer and the yield were generally higher for *A. niger* Δ*gaaB-gaaA* than for *A. niger* Δ*gaaB* when grown at pH 3 or 4 in either medium (Table 4). At pH 5 in *A. nidulans* minimal medium, the final L-galactonate titer 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* Δ*gaaB-gaaA* was higher than for *A. niger* Δ*gaaB* also at pH 5 (Table 4).

Transcription of putative transporter genes in *A. niger* Δ*gaaB*. The relative transcript levels of three genes which have been identified as possible transporters of D-galacturonate (An07g00780, An14g04280, and An03g01620, [12]) were assessed in *A. niger* ATCC 1015 and *A. niger* Δ*gaaB* 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 ATCC 1015 within 3 h of the transfer, whereas the induction of An07g00780 was only seen 24 h after the transfer. In contrast, no induction of An14g04280 was observed in *A. niger* Δ*gaaB*. Transcription of An03g1620 and An07g00780 in *A. niger* Δ*gaaB* 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 (Fig. 2 to 4). This confirmed that D-galacturonate was still taken up in the deletion strains, as was also the case when either the D-galacturonate reductase genes (*gar1* or *gaaA* in *T. reesei* and *A. niger*, respectively [14]) or the 2-keto-3-deoxy-L-

TABLE 2 Relative expression of *gaaA* in *A. niger* ATCC 1015, Δ*gaaB*, and Δ*gaaB-gaaA* strains[a]

Time (h)	Relative expression of *gaaA* (avg ± SEM [$n = 3$])		
	ATCC 1015	Δ*gaaB* strain	Δ*gaaB-gaaA* strain
0	0.2 ± 0.0	0.4 ± 0.0	14.0 ± 0.0
3	3.6 ± 0.6	0.1 ± 0.0	16.6 ± 0.6
6	2.6 ± 0.1	0.1 ± 0.0	9.5 ± 0.6
24	0.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.2

[a] The relative expressions of *gaaA* in *A. niger* ATCC 1015, Δ*gaaB*, and Δ*gaaB-gaaA* strains grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH 3.0 are presented.

TABLE 3 L-Galactonate production rates for *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* strains[a]

Time interval (h)	Avg L-galactonate production rate (g liter^{-1} h^{-1}) ± SEM ($n = 3$)[b]	
	Δ*gaaB* strain	Δ*gaaB-gaaA* strain
0–24	0.048 ± 0.001^{A}	0.070 ± 0.002^{B}
24–48	0.064 ± 0.001^{A}	0.075 ± 0.002^{B}
48–78	0.046 ± 0.000^{A}	0.054 ± 0.002^{A}

[a] The L-galactonate production rates were determined for *A. niger* Δ*gaaB* and Δ*gaaB-gaaA* strains grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3.0.
[b] Values in the same row with different superscript letters differed significantly ($P < 0.05$).

TABLE 4 L-Galactonate production at 144 h by *A. niger* Δ*gaaB* and the Δ*gaaB* strain overexpressing *gaaA* (Δ*gaaB-gaaA*)[a]

Medium	Initial pH	Strain	Mean amt of L-GalA (g liter^{-1}) ± SEM	Conversion (g g^{-1}) of L-GalA/D-GalUA$_{initial}$	Yield (g g^{-1}) of L-GalA/D-GalUA$_{consumed}$
A. nidulans MM	5	Δ*gaaB*	4.1 ± 0.2	0.20	0.82
	5	Δ*gaaB-gaaA*	4.1 ± 0.3	0.20	0.97
	4	Δ*gaaB*	7.2 ± 0.8	0.35	0.95
	4	Δ*gaaB-gaaA*	7.8 ± 0.4	0.38	0.97
	3	Δ*gaaB*	6.3 ± 0.1	0.31	0.86
	3	Δ*gaaB-gaaA*	8.7 ± 0.2	0.43	1.00
Modified Vogel medium	4	Δ*gaaB*	4.2 ± 0.1	0.41	0.70
	4	Δ*gaaB-gaaA*	5.0 ± 0.1	0.49	0.75
	3	Δ*gaaB*	4.9 ± 0.1	0.47	0.70
	3	Δ*gaaB-gaaA*	6.2 ± 0.3	0.59	0.82

[a] L-Galactonate (L-GalA) production at 144 h by *A. niger* Δ*gaaB* and the Δ*gaaB* strain overexpressing *gaaA* (Δ*gaaB-gaaA*) was determined in buffered *A. nidulans* minimal medium (MM) with 20 g of D-galacturonate liter^{-1} and 5 g of D-xylose liter^{-1} and in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} in flasks at an initial pH of 3, 4, or 5. Mean values are shown (n = 3). The conversion and yield on D-galacturonate (D-GalUA) are also shown.

galactonate aldolase genes, *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 of L-galactonate liter^{-1} h^{-1}) as previously reported for the conversion to keto-deoxy-L-galactonate (0.10 to 0.14 g liter^{-1} h^{-1} [20]) but was faster than the conversion to galactarate (0.024 to 0.046 g liter^{-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 of L-galactonate liter^{-1} h^{-1}) was much slower than the conversion to keto-deoxy-L-galactonate (0.27 to 0.33 g liter^{-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* Δ*lgd1* and 0.7 to 0.9 g g^{-1} for *A. niger* Δ*gaaB*. Thus, the yields were only slightly lower than the theoretical yield (1.0 g of L-galactonate g of D-galacturonate^{-1}) but still indicated that some of either the D-galacturonate or the produced L-galactonate was 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 measurable production of biomass from D-galacturonate in these strains.

TABLE 5 Relative expression of putative transporters An07g00780, An14g04280, and An03g01620 in *A. niger* ATCC 1015 and Δ*gaaB* strains[a]

Putative transporter	Time (h)	Avg relative expression ± SEM (n = 3): ATCC 1015	Avg relative expression ± SEM (n = 3): Δ*gaaB* strain
An07g00780	0	0.4 ± 0.0	0.1 ± 0.0
	3	0.1 ± 0.0	ND
	6	0.3 ± 0.0	0.2 ± 0.1
	24	1.1 ± 0.3	1.9 ± 1.0
An14g04280	0	0.0 ± 0.0	0.0 ± 0.0
	3	2.1 ± 0.2	0.1 ± 0.0
	6	0.9 ± 0.0	0.1 ± 0.0
	24	0.1 ± 0.0	0.1 ± 0.0
An03g01620	0	0.0 ± 0.0	0.0 ± 0.0
	3	2.0 ± 0.5	3.4 ± 0.4
	6	0.3 ± 0.0	0.2 ± 0.1
	24	0.0 ± 0.0	0.0 ± 0.0

[a] The relative expression of putative transporters An07g00780, An14g04280, and An03g01620 in *A. niger* ATCC 1015 and the Δ*gaaB* strain grown in flasks in modified Vogel medium with 10 g of D-galacturonate liter^{-1} and 2 g of D-xylose liter^{-1} at an initial pH of 3. ND, no data.

Although the production of both L-galactonate and keto-deoxy-L-galactonate require NADPH as a cofactor for the D-galacturonate reductase, L-galactonate production was more dependent on the addition of D-xylose as a cosubstrate (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 of 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 of L-galactonate g biomass^{-1}) in the corresponding strains (20). After provision of additional cosubstrate to *A. niger* Δ*gaaB* 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 ~2.86 times the dry biomass (10), the average intracellular concentration of L-galactonate was ~20 g liter^{-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 *T. reesei gar1* (10) and the *A. niger gaaA* (13) D-galacturonate reductases. The K_m for L-galactonate of *T. reesei* gar1 is 4 mM (0.8 g liter^{-1}) (10), which is much lower than the intracellular L-galactonate concentrations observed. Thus, the 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 the uptake of the substrate, about which little is known in filamentous fungi. Intracellular D-galacturonate was, however, only observed in *A. niger* and not in *T. reesei*.

In contrast to keto-deoxy-L-galactonate production (20), L-galactonate production was more efficient in *T. reesei* than in *A.*

niger at pH 5.5, producing higher titers at higher rates (Fig. 2). *T. reesei* was also found to be more effective than *A. niger* in the production of galactarate (14), and these results confirm that *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 is it tolerant to a very low culture pH.

Low galactarate production by *A. niger* Δ*gaaA-udh* was attributed to subsequent metabolism of the galactarate (14). The metabolism of L-galactonate appeared negligible (Fig. 4) or limited (Fig. 2) in *A. niger* Δ*gaaB*; instead, L-galactonate production by *A. niger* was found to be pH dependent, with the highest production rates and titers 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, the production of L-galactonate by *A. niger* Δ*gaaB* was as good as that of *T. reesei* Δ*lgd1* at pH 5.5. At a low extracellular pH, more of the product is protonated to L-galactonic acid (pKa ~3.5), creating a greater difference in concentration between the dissociated intra- and extracellular L-galactonate pools. If the protonated organic acid is not reimported into 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 liter^{-1} h^{-1} [20]) or galactarate at pH 5.0 (0.21 to 0.46 g liter^{-1} h^{-1} [14]) than were observed during L-galactonate production at any pH (0.04 to 0.15 g liter^{-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 the *A. niger* ATCC 1015, CBS120.49, and Δ*gaaA* strains (12, 14). In ATCC 1015, the transcription of these three genes was induced simultaneously within 4 h of transfer to D-galacturonate, and the induction of *gaaB* and *gaaC* remained similar in *A. niger* Δ*gaaA* compared to ATCC 1015 (14). In the present study, we observed that *gaaA* was not induced in *A. niger* Δ*gaaB* even 6 h after exposure to D-galacturonate (Table 2), although transcription had increased after 24 h. In ATCC 1015, *gaaA* expression was already reduced after 24 h of 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 responses of *gaaA* and *gaaC* might be expected since these genes share a 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* Δ*gaaB*. The L-galactonate production rate was initially significantly ($P < 0.002$) higher in *A. niger* Δ*gaaB-gaaA* compared to *A. niger* Δ*gaaB* in flasks at pH 3 (Table 3), 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 metabolizable carbon source (in this case, D-xylose), even though it is generally described as constitutive. Thus, the 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 the *A. niger* Δ*gaaB-gaaA* and *A. niger* Δ*gaaB* strains were similar (Table 3). The initial improved 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 4). Interestingly, the benefit of overexpression of *gaaA* was pH dependent even though the *gaaA* expression was not (Kuivanen, unpublished), with the greatest benefit at pH 3, although smaller improvements in yield were also observed at higher pH values (Table 4).

D-Xylose was previously found to be a good cosubstrate 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 while *gaaA* expression was high in *A. niger* Δ*gaaB-gaaA* probably limited the improvement in L-galactonate production that 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* Δ*gaaB* and *A. niger* Δ*gaaB-gaaA* may indicate that the protein encoded by An14g04280 has a dominant role.

Despite the fact that production of L-galactonate with *A. niger* Δ*gaaB* required more investigation and additional strain development than with *T. reesei* Δ*lgd1*, *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 does *T. reesei*, which is unable to degrade pectin (20). Using the current *A. niger* Δ*gaaB* strain, the 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 cosubstrates (e.g., D-galactose, D-xylose, and L-arabinose) for the initial production of biomass and NADPH. A more gradual provision of cosubstrate 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 cosubstrate was consumed and for the Δ*gaaB-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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