Received Date : 14-Feb-2014

Revised Date : 19-May-2014

Accepted Date : 19-May-2014

Article type : Original Article

Hyaluronic acid production with *Corynebacterium glutamicum*: effect of media composition on yield and molecular weight

Running Title: Hyaluronic acid production with *C. glutamicum*

Jana Hoffmann and Josef Altenbuchner*

Institute of Industrial Genetics, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany

***Corresponding author**

Phone: +49 711 685 675 91,

Fax: +49 711 685 669 73

E-mail: josef.altenbuchner@iig.uni-stuttgart.de

Abstract

Aim: *Corynebacterium glutamicum* was tested as an alternative host for heterologous production of hyaluronic acid (HA).

Methods and Results: A set of expression vectors containing *hasA*, encoding HA synthase from *Streptococcus equi* subsp. *zooepidermicus*, alone or in combination with genes encoding enzymes for HA precursor production (*hasB*, *hasC*, *glmU* from *Pseudomonas putida* KT2440) or bacterial haemoglobin (*vgb* from *Vitreoscilla* sp.) was constructed. Recombinant *C. glutamicum* strains were cultivated in two different minimal media, CGXII and MEK700. Hyaluronic acid was isolated from the culture broth by ethanol precipitation or ultrafiltration. Analyses of the isolated HA revealed that overall production was higher in CGXII medium (1,241 mg l^{-1}) than in MEK700 medium (363 mg l^{-1}) but molecular weight of the product was higher in MEK700 (>1.4 MDa) than in CGXII (<270 kDa). Coexpression of *hasB*, *hasC* or *glmU* had no effect on HA yield and did not improve molecular weight of the product. Coexpression of *vgb* lowered HA yield about 1.5-fold and did not affect molecular weight of the product. Microscopy of negative stained cultures revealed, that *C. glutamicum* produces no distinct HA capsule.

Conclusions: Regulation of cell growth and gene expression level of *hasA* are reasonable starting-points for controlling the molecular weight of HA produced by *C. glutamicum*.

Significance and Impact of the study: *C. glutamicum* has a great potential as an alternative production host for HA. The fact that *C. glutamicum* produces no distinct HA capsule facilitates HA isolation and improves overall yield.

Introduction

Hyaluronic acid (HA) is a linear high molecular weight glycosaminoglycan consisting of repeating units of β-1,3 and β-1,4 linked glucuronic acid and *N*-acetylglucosamine (Figure 1). HA naturally occurs in vertebrate tissues, especially in the extracellular matrix, synovial fluid, umbilical cord, and vitreous humor of the eye, where it fulfils diverse structural and biological functions (Liu *et al.* 2011). Besides, HA is a capsule component of some pathogenic microorganisms, *e. g. Pasteurella multocida* (DeAngelis *et al.* 1998) and *Streptococcus* sp. (Izawa *et al.* 2009).

Due to its unique physicochemical and rheological properties, particularly the exceeding water binding capacity and viscoelasticity, HA is an excellent lubricant and shock absorber and hence a valuable material for numerous medical and cosmetic applications (Kogan *et al.* 2007; Necas *et al.* 2008). As a key material in ophthalmic surgery, HA functions as tissue protector, spacer, substituent for lost vitreous fluid or component of plastic intraocular lenses for implantation. Viscosupplementation of joints affected by arthritis is the second major application of HA. Moreover, HA has proven to be a useful material in nanobiotechnology. HA and chitosan based hollow microcapsules filled with drugs, peptides or nucleic acids can shield the agents from degradation and thereby promote their targeted delivery (Cui *et al.* 2011).

Highly pure HA as a clinical product was first prepared from rooster combs (Balasz 1979). To date, the extraction from animal waste is still an important source of HA. However, the established processes generally suffer from low yields and high polydispersity of the isolated product (Boeriu *et al.* 2013). Since the 1980s, the increasing demand of HA was additionally supplied by fermentation of natural HA producers, *i. e.* group A and C streptococci (Woolcock 1974). Although process and strain optimization led to yields of 6-7 g l^{-1} (Kim *et al.* 1996), lactic acid bacteria have a significant disadvantage. Especially in defined cultures

phages can hamper fermentation and the phage-host interactions are not sufficiently understood to completely overcome the infections during fermentations (Mahony *et al.* 2012).

HA synthesis (Figure 2) and transport is catalyzed by the HA synthases. In *Streptococcus equisimilis* the HA synthase, encoded by *hasA*, is an integral membrane protein that catalyzes the polymerization of UDP-glucuronic acid and UDP-N-acetylglucosamine and simultaneous translocation of the growing HA-chain across the membrane (Hubbard *et al.* 2012). Most non HA producing bacteria can easily be transformed into HA producers by introducing the *hasA* gene. Until now, only three species not belonging to the lactic acid bacteria were tested as alternative hosts for HA production. *Escherichia coli* (Yu and Stephanopoulos 2008; Mao *et al.* 2009) and *Agrobacterium* sp. (Mao and Chen 2007) were optimized for HA production and at least *E. coli* produced considerable amounts of HA (Production with *Agrobacterium* sp.: 0.3 g l^{-1}; production with *E. coli*: 3.8 g l^{-1}). Yet, the Gram negative hosts generally comprise the disadvantage of endotoxin production and thus a potential contamination of the product. HA production in the multigram range (up to 6.8 g l^{-1}) was also achieved with the Gram positive, generally recognized as safe (GRAS) bacterium *Bacillus subtilis* (Widner *et al.* 2005; Jia *et al.* 2013).

Corynebacterium glutamicum is the most important GRAS microorganism for industrial amino acid production (Eggeling and Bott 2005). The great economical potential of *C. glutamicum* impelled more than fifty years of research and led to an extensive knowledge of the corynebacterial metabolism and regulatory networks (Wittmann and Heinzle 2008; Buchinger *et al.* 2009; Teramoto *et al.* 2011; Becker and Wittmann 2012). To date, various genetic tools are available for modification of *C. glutamicum* and new tools are being continuously developed (Nešvera and Pátek 2011). Besides amino acids, *C. glutamicum* is also used for the biosynthesis of *e. g.* pantothenic acid (Chassagnole *et al.* 2003), organic

acids (Wieschalka *et al.* 2013), carotenoids (Heider *et al.* 2012) and biofuels (Inui *et al.* 2004; Smith *et al.* 2010; Blombach *et al.* 2011) and it has a great potential for the production of other economically relevant substances.

This study examined the applicability of *C. glutamicum* for the production of hyaluronic acid. A modular plasmid-based expression system was constructed for the heterologous expression of *hasA* along with other genes for enhanced HA production. HA was produced in two different minimal media and the isolated product was characterized by gel permeation chromatography, hyaluronidase digestion and microscopy.

Materials and methods

Materials and standard procedures

Pure chemicals were purchased from Sigma-Aldrich Labor Chemie GmbH (Steinheim, Germany) or VWR International GmbH (Darmstadt, Germany). Isolation of genomic DNA, DNA digestion, ligation and transformation procedures were performed according to Sambrook *et al.* (1989). Plasmid and PCR purification kits were provided by Qiagen (Hilden, Germany). Brain heart infusion, european agar, tryptone and yeast extract were purchased from BD (Heidelberg, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). T4 DNA ligase was purchased from Roche (Grenzych-Wyhlen, Germany). Oligonucleotides were synthesized by Eurofins MWG (Ebersberg, Germany). Phusion Hot Start DNA polymerase was purchased from Thermo Scientific (Dreieich, Germany).

Bacterial strains, media and culture conditions

Escherichia coli JM109 (*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *sup*E44 *relA*1 λ^- Δ(*lac-proAB*) [F' *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) (Yanisch-Perron *et al.* 1985) was used for plasmid

propagation and preparation. The *E. coli* strains were cultivated in Luria-Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl) supplemented with 50 mg l^{-1} kanamycin or 100 mg l^{-1} ampicillin at 37°C.

Corynebacterium glutamicum ATCC 13032 (Abe *et al.* 1967) was cultivated in LB medium, brain heart infusion sorbitol (BHIS) medium (37 g l^{-1} brain heart infusion powder, 91 g l^{-1} sorbitol), MEK700 minimal medium (see below) or CGXII minimal medium (see below) at 30°C.

The modified MEK700 minimal medium (Onaca *et al.* 2007) was prepared by mixing several separately sterilized solutions. 824 ml deionized water was mixed with 50 ml phosphate buffer (20 g l^{-1} KH_2PO_4, 70 g l^{-1} $Na_2HPO_4 \cdot 2\ H_2O$, pH 7.1), 10 ml SL100 salt solution (100 g l^{-1} $(NH_4)_2SO_4$, 20 g l^{-1} $MgSO_4$, 1 g ammonium iron(III) citrate, 100 ml trace element solution SL6, see below), 1 ml calcium nitrate solution (50 g l^{-1} $Ca(NO_3)_2 \cdot 4\ H_2O$). Furthermore, the medium was supplemented with final concentrations of 0.1% (w/v) casamino acids, 2 mg l^{-1} biotin, 20 mg l^{-1} thiamine, and 0.2-4% (w/v) D-glucose and the volume was adjusted to 1 l with deionized water. The SL6 trace element solution contained 100 mg l^{-1} $ZnSO_4 \cdot 7\ H_2O$, 30 mg l^{-1} $MnCl_2 \cdot 4\ H_2O$, 300 mg l^{-1} H_3BO_3, 200 mg l^{-1} $CoCl_2 \cdot 6\ H_2O$, 10 mg l^{-1} $CuCl_2 \cdot 2\ H_2O$, 20 mg l^{-1} $NiCl_2 \cdot 6\ H_2O$, 30 mg l^{-1} $Na_2MoO_4 \cdot 2\ H_2O$.

The modified CGXII minimal medium (Keilhauer *et al.* 1993) was prepared by dissolving 5 g $(NH_4)_2SO_4$, 5 g urea, 21 g 3-morpholinopropanesulfonic acid (MOPS), 1 g K_2HPO_4, and 1 g KH_2PO_4 in deionized water. The pH was adjusted to 7.0 with NaOH/HCl, the volume was adjusted to 896 ml with deionized water and the solution was autoclaved for sterilization. After cooling, 250 mg l^{-1} $MgSO_4$, 10 mg l^{-1} $CaCl_2$, 1 ml l^{-1} trace element solution (see below), 0.2 mg l^{-1} biotin, 0.3 mg l^{-1} protocatechuic acid, and 0.2-4% (w/v) D-glucose were added and the volume was adjusted to 1 l with sterile deionized water. The trace element solution consisted of 16.4 g l^{-1} $FeSO_4 \cdot 7\ H_2O$, 100 mg l^{-1} $MnSO_4 \cdot H_2O$, 200 mg l^{-1} $CuSO_4$, 1 g l^{-1}

$ZnSO_4 \cdot 7\ H_2O$, 20 mg l^{-1} $NiCl_2 \cdot 6\ H_2O$. For dissolving the components, the pH was adjusted to 1 with HCl.

Plasmid-carrying *C. glutamicum* strains were cultivated in duplicate in 15 or 25 ml MEK700 or CGXII minimal medium supplemented with 50 mg l^{-1} kanamycin (100 or 250 ml Erlenmeyer flasks) at 30°C and 200 rpm starting from an initial optical density at 600 nm (OD_{600}) of 0.05. 1 mmol l^{-1} β-D-1-thiogalactopyranoside (IPTG) was added to one culture when the OD_{600} reached values between 0.25 and 0.3. The second culture was left uninduced as a control. The cultures were further incubated at 30°C and 200 rpm for 24-120 h.

Cell dry mass determination

C. glutamicum wild type was cultivated for 24 h at 30°C and 200 rpm in 100 ml MEK700 or CGXII medium starting with an initial OD_{600} of 0.05. The final OD_{600} of the cultures was measured four times. Six samples (each 10 ml) were centrifuged in glass test tubes (4,000 *g*, 20 min) and the supernatants were discarded. The cells were resuspended in 1 ml H_2O and transferred to 5 ml polystyrene test tubes (Sarstedt, Nümbrecht, Germany). The glass test tubes and the pipette tips were washed twice with 0.5 ml H_2O and the washing water was also transferred to the polystyrene test tubes. The samples were centrifuged (4,000 *g*, 20 min) and the cell pellets were dried for four days in an exsiccator over $CaCl_2$. Afterwards, the pellets were further dried in an oven for two days at 60°C. The tubes with the dried cells were weighed and cell dry masses (CDM) were determined by subtraction of the empty weight of each tube. The calculated CDM to OD_{600} ratios were 0.34 g l^{-1} OD_{600}^{-1} in MEK700 medium and 0.309 g l^{-1} OD_{600}^{-1} in CGXII medium.

Electroporation of *C. glutamicum*

Electroporation of *C. glutamicum* was performed as described by van der Rest *et al.* (1999) with some modifications. The cells were grown overnight in 5 ml BHIS medium using long glass test tubes at 30°C and 200 rpm. The main culture (100 ml BHIS, 1 l Erlenmeyer flask) was inoculated with the overnight culture (initial OD_{600} of 0.2) and grown for 3.5 h at 30°C and 200 rpm (final OD_{600} of 2.0). The cells were harvested by centrifugation (4,000 *g*, 20 min, 4°C), washed three times with 20 ml ice-cold 20% (v/v) glycerol (centrifugation 3,000 *g*, 15 min, 4°C) and resuspended in 4 ml ice-cold 20% (v/v) glycerol. 100 µl aliquots of the cell suspension were frozen at -70°C until use. For electroporation, the cells were thawed slowly on ice, mixed with 200 ng plasmid DNA and incubated on ice for 15 min. Electroporation was performed in cuvettes (2 mm interelectrode distance) with the Gene Pulser I (Bio-Rad Laboratories, München, Germany) with instrument settings 2.5 kV, 25 µF and 200 Ω. After electroporation, 1 ml BHIS was added to the cuvette. The cell suspension was then transferred into a glass test tube and incubated for 6 min at 46°C in a water bath. Afterwards, the test tubes were incubated for 1 h at 30°C on a rotator. The cells were plated on LB agar with 50 mg l^{-1} kanamycin and incubated at 30°C for 48 h.

Plasmid construction

hasA from *Streptococcus equi* subsp. *zooepidemicus* MGCS10565 (Gene ID 6760576) was synthesized by Genscript (Piscataway, NJ, USA). The internal *Hin*dIII site of *hasA* was removed. An *Nde*I site was introduced at the 5' end and *Xba*I and *Hin*dIII sites at the 3' end of *hasA*. The sequence was adjusted to the *E. coli* codon usage with JCat (Grote *et al*. 2005). The gene was provided by Genscript as an insert in pUC57 (pUC57*hasA*, Table S1). *hasA* was excised from pUC57*hasA* with *Nde*I and *Hin*dIII and integrated into pJeM1 to construct pJH122.1 (Table S1).

All *C. glutamicum* hyaluronic acid production plasmids stem from the shuttle vector pJOE7706.1 (Table S1). pJOE7706.1 is derived from pVWEx1 (Table S1). The polylinker sequence downstream of the *tac* promotor in pVWEx1 was replaced by *eGFP* containing a N-terminal his-tag sequence, the pACYC177 replication region was replaced by the pBR322 (Table S1) replication region and several small dispensable parts of the vector backbone were deleted by restriction enzyme digestion and religation.

The genes used for the construction of the hyaluronic acid production vectors were amplified by PCR with the oligonucleotides given in Table S2. pJH122.1 was used as template for amplification of *hasA*. Chromosomal DNA of *Pseudomonas putida* KT2440 (Franklin *et al.* 1981) was used as template for amplification of *hasB* (Gene ID1042906), *hasC* (Gene ID 1046744), and *glmU* (Gene ID 1045333). pUC8:16 (Table S1) was used as template for amplification of *vgb*. Blunt-end PCR products were inserted into pJOE4786.1 (digested with *Eco*RV) for DNA sequencing. Fragments with correct nucleotide sequences were excised from pJOE4786.1 (*hasA* with *Bsr*GI/*Nde*I, all other genes with *Spe*I/*Nhe*I) and successively integrated into pJOE7706.1 (Figure 2). Restriction sites used for plasmid construction that are not part of the oligonucleotides used for amplification of the particular gene originate either from PCR products or pJOE4786.1.

Isolation of HA from culture broth by ultrafiltration

The cultures (1 or 10 ml) were harvested by centrifugation (16,000 *g*, 5 min or 3,500 *g*, 10 min), the supernatants were isolated and mixed with 1/10 volume of 100% (w/v) trichloroacetic acid (TCA) in order to precipitate any protein (30 min on ice). The samples were centrifuged (16,000 *g*, 30 min), the supernatants were isolated and applied for ultrafiltration.

The 0.5 ml ultrafiltration devices Amicon 30k (Merck Millipore, Darmstadt, Germany) were applied according to the manufacturer´s protocol. The 1 ml probes were loaded stepwise onto the ultrafiltration device. After centrifugation (16,000 *g*, 8 min), the samples were washed three times by addition of 400 µl H_2O and subsequent centrifugation. The isolated HA was eluted with 400 µl H_2O. The exact volume of each sample was determined after elution.

Isolation of HA from culture broth by ethanol precipitation

10 ml TCA-precipitated culture supernatants (see above) were mixed with 20 ml 99.8% (v/v) ethanol and stored at -20°C overnight. The samples were centrifuged (3,500 *g*, 30 min) and the supernatants were discarded. The pellets were washed three times with 10 ml 99.8% (v/v) ethanol and afterwards air dried. The dried pellets were resolved in 1.5 ml H_2O and the obtained turbid solutions were cleared by centrifugation (16,000 *g*, 10 min).

Quantification of HA by carbazole reaction

The modified carbazole reaction (Bitter and Muir 1962) was performed as follows. First, a calibration curve with seven different HA concentrations (10 – 5,000 mg l^{-1}) was prepared. 1 ml portions of 25 mmol l^{-1} sodium tetraborate in sulfuric acid were pipetted into 2 ml reaction tubes and cooled in a microtube cooling rack (precooled at -20°C). 167 µl of each HA sample were carefully layered onto the precooled acid. The reaction tubes were inverted once for mixing and cooled again for some minutes. The probes were then mixed by vortexing and left to stand in the cooling rack until no more warmth was produced. The probes were incubated in a boiling water bath for 10 min and afterwards cooled on ice. 33 µl of 0.125% (v/w) carbazole in 99.8% (v/v) ethanol were added, the samples were mixed and boiled again in a water bath for 15 min. After cooling, the absorption was read

photometrically at 530 nm (Ultrospec 300, Amersham Pharmacia Biotech, Nümbrecht, Germany). The linear equation of the obtained calibration curve was used for calculation of the HA contents of samples prepared from the *C. glutamicum* cultures.

Digestion of produced HA with hyaluronidase

Hyaluronidase (Type I-S, from bovine testes, E.C. 3.1.1.35) was purchased from Sigma Aldrich (Steinheim, Germany). HA digestion by hyaluronidase (HAase) was measured turbidimetrically. For calibration, 500 µl of five different HA solutions (80 – 320 mg l^{-1}) in phosphate buffer (300 mmol l^{-1} $NaH_2PO_4 \cdot H_2O$, pH 5.35 at 37°C) were mixed with 500 µl enzyme diluent (20 mmol l^{-1} $NaH_2PO_4 \cdot H_2O$, 77 mmol l^{-1} NaCl, 0.01% (w/v) bovine serum albumin, pH 7.0 at 37°C). 100 µl of each mixture were pipetted into 900 µl acetic albumin solution (24 mmol l^{-1} sodium acetate, 79 mmol l^{-1} acetic acid, 0.1% (w/v) bovine serum albumin, pH 3.75 at 25°C) and incubated at room temperature for 30 min. The turbidity was measured at 600 nm in a photometer. A calibration curve was calculated and its linear equation was applied for the quantification of HA.

For digestion of HA produced by *C. glutamicum*, 250 µl of HA solutions (300 mg l^{-1} in phosphate buffer, content determined by carbazole reaction) were incubated at 37°C for 10 min. Then, 250 µl of HAase solution (24 mg l^{-1} HAase in enzyme diluent, prewarmed at 37°C) were added and the samples were incubated at 37°C. After 20 min, 100 µl of the samples were mixed with 900 µl acetic albumin solution and incubated at room temperature for 30 min. The absorption was read at 600 nm in a photometer. HA produced by *Streptococcus equi* (300 mg l^{-1} in phosphate buffer, purchased from Sigma Aldrich, Steinheim, Germany) was used as a control. For each sample, a parallel probe was prepared,

where enzyme diluent without HAase was added. The HA contents of the samples with HAase were subtracted from the HA contents of the samples without HAase to calculate the amount of digested HA.

Fluorescence measurement of eGFP

Cultures of *C. glutamicum* pJOE7706.1 were diluted with the medium used for cultivation to an OD_{600} of 0.1. Fluorescence (485 nm excitation wavelength, 535 nm emission wavelength) of 100 µl of the diluted cultures was measured by a Genios microplate reader in fluorescense top measurement mode (Tecan, Crailsheim, Germany). The fluorescence of 100 µl culture medium was also measured and the obtained value was subtracted from the fluorescence value of the culture samples.

Glucose Determination

The quantification of glucose in medium supernatants was performed with glucose oxidase (EC 1.1.3.4), horseradish peroxidase (EC 1.11.1.7), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (Werner *et al.* 1970).

Microscopy

5 µl culture or supernatants of centrifuged (16,000 g, 8 min) cultures were mixed with 5 µl H_2O and 5 µl nigrosin (100 g l^{-1}) on a microscope slide. The mixture was streaked out with a cover slip and afterwards air-dried. Preparations were investigated by phase contrast microscopy (Axioplan microscope, Carl Zeiss, Oberkochen, Germany) with a 100× oil-immersion objective. The Axiocam MRm camera and the Axiovision Software (Carl Zeiss, Oberkochen, Germany) were used for documentation.

Molecular weight analysis by GPC

GPC analyses were performed with an HPLC system (Merck Hitachi, Darmstadt, Germany) consisting of L-7100 pump, L-7000 interface module, Rheodyne sample injector 9725i with 100 µl sample loop, D-7000 HPLC system Manager Software, and RI detector 8100 (Bischoff, Leonberg, Germany). Suprema 300 Å column (20 µm, 8 × 300 mm), Suprema 10,000 Å column (20 µm, 8 × 300 mm), and Suprema guard column (20 µm, 8 × 50 mm) (PSS Polymer Standard Service, Mainz, Germany) were used for chromatographic separation at room temperature. The mobile phase was 50 mmol l^{-1} NaCl with a flow rate of 1 ml min^{-1}. The system was calibrated with dextranes of different molecular masses (2 g l^{-1}, dissolved in 50 mmol l^{-1} NaCl, injection volume 100 µl). HA samples obtained by ethanol precipitation were equilibrated with 50 mmol l^{-1} NaCl by Amicon 30k ultrafiltration prior to analysis (HA concentration 2 g l^{-1}, injection volume 50 or 100 µl).

Results

Construction of an expression platform for heterologous HA production in *C. glutamicum*

Previous studies revealed that improved precursor or oxygen supply accomplished by overexpression of *hasB*, *hasC*, *glmU* (Figure 2) or *vgb* (bacterial haemoglobin) enhanced overall yield and/or molecular weight of HA produced by *B. subtilis*, *E. coli*, *Agrobacterium* sp. or *Streptococcus thermophilus* (Widner *et al.* 2005; Chien and Lee 2007; Mao and Chen 2007; Yu and Stephanopoulos 2008; Sheng *et al.* 2009; Prasad *et al.* 2010; Izawa *et al.* 2011). For heterologous HA production with *C. glutamicum*, seven hyaluronic acid production vectors containing either *hasA* alone or in combination with *hasB*, *hasC*, *glmU* or *vgb* were constructed by serial integration of the respective DNA-fragments into pJOE7706.1 (Figure

3). *C. glutamicum* was transformed with the plasmids by electroporation and the IPTG-dependent HA production was investigated in two different minimal media, *i. e.* MEK700 and CGXII. The two media differ in their buffer and nitrogen contents. Compared to MEK700, CGXII contains MOPS, urea, a larger quantity of ammonium sulphate, and it lacks casamino acids (see material and methods).

Production of hyaluronic acid by *C. glutamicum* in minimal medium in batch cultures

A preliminary test with *C. glutamicum* pJH197.1 revealed that the initial glucose concentration influenced HA production, CDM and also gene expression levels in both media (Figure 4, Table 1). Uninduced MEK700 cultures reached a maximum CDM of 3.06 g l^{-1}, which were already obtained with 0.8% (w/v) glucose after 48 h of cultivation. Higher glucose concentrations did not further increase the CDM of the cultures (Figure 4 a). In contrast, the CDM of the uninduced CGXII cultures increased continuously, when higher glucose concentrations were applied and reached a maximum of 8.1 g l^{-1} (Figure 4 b). The induced cultures of *C. glutamicum* pJH197.1 with more than 0.5% (v/w) glucose reached lower CDM than the uninduced cultures in both media (Figure 4 a, b). HA production and CDM of the induced MEK700 cultures decreased when more than 0.8% (w/v) glucose were applied (Figure 4 a). In addition, it was observed that HA production of the uninduced MEK700 cultures increased when higher glucose concentrations were applied (Figure 4 a). This phenomenon was less pronounced in the CGXII cultures with 0.2-1% glucose, only the uninduced culture with 4% glucose showed a higher production (Figure 4 b). The highest HA production was measured in induced CGXII cultures with 4% (w/v) glucose (Figure 4 b). The data of the wild type controls indicated, that *C. glutamicum* produces a significant amount of a high molecular weight substance in both media that interferes with the carbazole reaction

(Figure 4 c, d). This substance was not measured, when the media were subjected to ultrafiltration and afterwards measured by the carbazole assay before cultivation of *C. glutamicum* (data not shown). Fluorescence measurements of cultures expressing *eGFP* with the basic vector pJOE7706.1 revealed a lower fluorescence when the cells were cultivated in MEK700, indicating that gene expression from the *tac* promoter is weaker in MEK700 medium (Table 1).

Initial glucose concentrations of 0.5% (w/v) in MEK700 medium and 1% (w/v) in CGXII medium were applied for investigation of the HA production of the different plasmid-carrying *C. glutamicum* strains (Figure 5). After 48 h of cultivation, HA contents of all induced cultures were considerably higher in CGXII medium (up to 504 ± 20 mg l^{-1}) than in MEK700 medium (up to 287 ± 28 mg l^{-1}) (Figure 5 a, b). Glucose was completely consumed in all cultures (data not shown). The pH values of the MEK700 decreased and the pH values of the CGXII cultures increased after 48 h of cultivation (Table 2).

The addition of 4% (w/v) glucose and further incubation for 72 h enhanced the HA production of the induced cultures about 2.5-fold in CGXII medium (up to 1,241 ± 227 mg l^{-1}) and about 1.2-fold in MEK700 medium (up to 363 ± 17 mg l^{-1}) (Figure 5 c, d). After overall 120 h of cultivation, also the uninduced cultures with plasmids produced considerable amounts of HA (up to 108 ± 2 mg l^{-1} in MEK700, up to 389 ± 42 mg l^{-1} in CGXII). The CDM in MEK700 medium only slightly increased after the second glucose feeding (Figure 5 a, c). In contrast, the CDM of the CGXII cultures at least doubled after the addition of 4% (w/v) glucose (Figure 5 b, d). Regarding the HA production of the induced cultures, only negligible differences between the strains with pJH174.1, pJH181.3, pJH195.2, pJH196.2, and pJH197.1 were measured in both media, indicating that coexpression of *hasB*, *hasC*, or *glmU* had no effect on the HA production of *C. glutamicum* under the tested conditions.

However, *C. glutamicum* pJH182.1, which coexpressed *hasA* and *vgb* produced a lower amount of HA in both media after 48 h (220 ± 23 mg l^{-1} in CGXII, 126 ± 8 mg l^{-1} in MEK700) as well as 72 h after the second glucose feeding (718 ± 18 mg l^{-1} in CGXII, 160 ± 24 mg l^{-1} in MEK700) (Figure 5). Hence, coexpression of *vgb* and *hasA* lowered the HA production of *C. glutamicum*. *C. glutamicum* pJH182.1 also showed a difference to the other cultures in respect of the reached CDM. Induced cultures coexpressing *hasB*, *hasC* or *glmU* in addition to *hasA* reached lower CDM than the induced cultures while the induced cultures coexpressing *vgb* and *hasA* reached CDM comparable to the uninduced cultures and rather behaved like the wild type cultures without plasmid (Figure 5). This effect could be observed especially when the cells were cultivated in CGXII medium (Figure 5 b, d).

The CDM data are reflected by the glucose consumption of the cultures after overall 120 h of cultivation. Glucose was completely consumed in uninduced CGXII cultures, CGXII wild type cultures, and induced *C. glutamicum* pJH182.1 cultures (data not shown). The remaining induced GCXII cultures consumed 45.54 ± 1.09 g l^{-1} glucose. The uninduced MEK cultures consumed 26.83 ± 1.60 g l^{-1} glucose, the induced MEK cultures consumed 21.30 ±2.42 g l^{-1} glucose, and the MEK wild type culture consumed 22.95 ± 1.35 g l^{-1} glucose. The corresponding substrate-specific HA yields are given in Table 3. The acidification of all MEK700 cultures was more pronounced after 120 h (Table 2). In CGXII medium, a strong acidification was only observed in the induced cultures (Table 2), in uninduced and wild type cultures neutral pH values were measured after 120 h (Table 2). Only the induced CGXII culture of *C. glutamicum* pJH182.1 behaved differently from the other cultures (pH 6.78 ± 0.05).

Characterization of the produced HA

The molecular weight of HA is an important quality parameter, because viscoelastic rheology and water binding capability of HA with low molecular weight are reduced compared to HA with high molecular weight (Liu *et al.* 2011). Analysis of the produced HA by GPC revealed that the molecular weight of the product was much higher, when the strains were cultivated and induced in MEK700 medium (Figure 6). Again, the differences between the different plasmid-carrying strains were less pronounced. Coexpression of *hasA*/*hasC* (pJH181.3), *hasA*/*hasC*/*hasB* (pJH183.2), and *hasA*/*glmU*/*hasC* (pJH197.1) reduced the molecular weight of the produced HA in MEK700 medium.

HA produced by *C. glutamicum* was sensitive to hyaluronidase digest (Figure 7). Turbidity of an acetic albumin solution caused by addition of HA was reduced comparably by factors of 2.19 (HA from *S. equi*, control), 2.7 (MEK700) and 2.39 (CGXII) when the samples were treated with HAase. High molecular weight HA produced in MEK700 medium led to the same turbidity as the standard HA from *S. equi*. It could be observed that although the same amount of HA was added to all samples, HA with lower molecular weight that was produced in CGXII medium formed lower turbidity with acetic albumin than HA with higher molecular weight (Figure 7).

C. glutamicum produces no distinct HA capsule

In streptococci HA is a natural component of the capsule (Izawa *et al.* 2009) and also strains that were artificially engineered for HA production can form HA capsules that can be visualized by negative staining with nigrosin (DeAngelis *et al.* 1998). The acidic stain is repelled by negative charged cell surfaces. Therefore, cells are not stained and appear as colourless structures on a dark background under the microscope. Bacterial capsules have a

dense gelatinous consistence and are also not penetrated by the dye. When present, capsules can be observed as light halos around cells in negative stained preparations.

Uninduced and induced *C. glutamicum* cultures and culture supernatants were stained with nigrosin and the preparations were checked by microscopy (Figure 8, S1). Preparations of uninduced cultures looked like preparations of wild type cultures and rather showed cell pairs or groups of cells than single cells (Figure 8, S1 a). Especially the V-forming cell pairs are typical for corynebacteria and are a result of their snapping mechanism of cell division (Letek *et al.* 2008). The cells of induced cultures looked like the uninduced cells, hence there are likely no distinct HA capsules around the cells (Figure 8 a, b, g, h; Figure S1 c, d, g, h). Furthermore, SDS-extraction of cells gave no detectable HA (data not shown). In addition, the preparations of the induced MEK700 culture of *C. glutamicum* pJH174.1 exhibited more unstained white areas than the preparation of the uninduced culture (Figure 8 a, b). These white spots could also be observed in preparations of cell free culture supernatants (Figure 8 c, d). When the supernatants were treated with hyaluronidase prior to preparation, the spots vanished in the preparation of the induced supernatant (Figure 8 e, f). Therefore, the white spots are most likely unstained HA droplets that were released to the medium by the induced cells. Comparable results were obtained with MEK700 cultures of *C. glutamicum* pJH181.3, pJH195.1, pJH196.2, and pJH197.1 (data not shown). In contrast, the preparation of the induced MEK700 culture of *C. glutamicum* pJH182.1 showed smaller HA droplets (Figure 8 g, h).

The HA droplets formed by *C. glutamicum* pJH174.1 in CGXII medium were very small and only detectable, when undiluted culture supernatant was stained (Figure S1 f). Preparation of CGXII cultures and culture supernatants of *C. glutamicum* pJH181.3, pJH195.2, pJH196.2, and pJH197.1 gave comparable results (data not shown). The HA droplets in the preparations of induced *C. glutamicum* pJH182.1 culture supernatants were hardly visible (Figure S1 j).

The cells cultivated in CGXII medium also showed no distinct HA capsules (Figure S1 c, d, g, h) and looked like the wild type cells (Figure S1 b).

Discussion

Hyaluronic acid synthesis competes for precursors that are also metabolized in glycolysis, pentose phosphate pathway or cell wall synthesis (Figure 2). For all tested hosts for heterologous HA production it was reported that the sole expression of *hasA* yielded only low amounts of HA and that the additional expression of genes for precursor supply enhanced yield and/or molecular weight in both natural and heterologous HA producers (Widner *et al.* 2005; Mao and Chen 2007; Yu and Stephanopoulos 2008; Sheng *et al.* 2009; Prasad *et al.* 2010; Izawa *et al.* 2011). In contrast to these previous reports, *C. glutamicum* produced considerable amounts of HA when only *hasA* was expressed. Moreover, the additional expression of *hasB*, *hasC* or *glmU* did not enhance overall production or molecular weight. This indicates that the precursor pool of UDP-glucuronic acid and UDP-N-acetylglucosamine was not limiting for HA production in *C. glutamicum* under the tested conditions.

The regulation of the central metabolism, mode of carbon catabolite repression and regulation of gene expression in *C. glutamicum* significantly differs from those in *E. coli* and *B. subtilis* (Eikmanns 2005; Moon *et al.* 2007; Schröder and Tauch 2010; Eikmanns and Blombach 2014). Hence, the principles derived from experiments with these organisms might not be readily transferable to *C. glutamicum.* This assumption is also substantiated by the effects caused by coexpression of the *Vitreoscilla* sp. haemoglobin gene *vgb*. Coexpression of *vgb* doubled HA production in *B. subtilis* (Chien and Lee 2007) but resulted in lower HA production in *C. glutamicum*. Generally, induced cultures of HA producing *C. glutamicum*

that did not coexpress *vgb* reached lower CDM than the uninduced cultures. This is a known phenomenon of HA production (Prasad *et al.* 2010) and a result of the competition of the energy-consuming HA synthesis with cell growth for the universal precursors glucose 6-phosphate and fructose 6-phosphate. HA producing *C. glutamicum* that coexpressed *vgb* reached higher CDM than the cultures without *vgb*. Hence, improved oxygen supply redirected the metabolic flux towards cell growth and energy generation and lowerd HA production in *C. glutamicum*. When *C. glutamicum* cells are deprived of oxygen, they stop growing but remain metabolically active and retain their ability to excrete significant amounts of organic acids from glucose even in minimal medium (Yamamoto *et al.* 2011). Some compounds, *e. g.* succinate, L-valine or xylitol can be efficiently produced under oxygen-deprivation conditions (Sasaki *et al.* 2010; Yamamoto *et al.* 2013; Hasegawa *et al.* 2013). Taking account of the results obtained by coexpression of *vgb*, production under oxygen-deprivation might also be a strategy for enhancing HA production with *C. glutamicum*.

The recombinant *C. glutamicum* strains produced considerably more HA in CGXII medium than in MEK700 medium. In addition, the CDM reached in CGXII were also significantly higher. In MEK700 medium HA production and CDM decreased when a glucose concentration of 4 % (w/v) was applied. A similar phenomenon was reported for HA producing cultures of *Lactococcus lactis* (Prasad *et al.* 2010). The effect was due to acidification of the culture broth and could be relieved by pH control in a bioreactor. After 48 h of cultivation of *C. glutamicum*, the pH values in MEK700 medium decreased, while the pH values in CGXII medium increased. CGXII medium contains urea that is cleaved by *C. glutamicum* urease resulting in release of alkalizing ammonia and carbon dioxide (Puskás *et al.* 2000; Beckers *et al.* 2004). Although urea can thus be utilized by *C. glutamicum*, growth is not affected, when urea is omitted in CGXII medium (Unthan *et al.* 2014) and here rather

fulfills the role of a buffering substance than a nitrogen source. After 120 h of cultivation, the acidification of the MEK700 cultures was more pronounced and also the induced CGXII cultured acidificated. The strong drop in pH value of the MEK700 medium is a probable reason for weaker cell growth and HA production. However, it cannot be excluded that the different types and quantities of the applied nitrogen sources also influenced HA production by *C. glutamicum*. Acidification is likely caused by organic acids, *e. g*. lactic acid or succinic acid that can be produced by *C. glutamicum* from glucose (Okino *et al*. 2005; Rados *et al*. 2014), yet this remains to be verified experimentally. *C. glutamicum* wild type produced a high molecular substance in both media that interfered with the carbazole assay. This substance is likely a carbohydrate that originates from the top layer of the cell envelope of *C. glutamicum* (Burkovski 2013). It might contain uronic acids but neutral sugars are also known to interfere with the carbazole reaction (Bitter and Muir 1962). Due to the fact that the background of the substance was relatively low in MEK700 medium, the high production of the uninduced MEK700 cultures must be due to leaky expression from the *tac* promoter.

Interestingly, the molecular weight of the HA produced in MEK700 medium was much higher than in CGXII medium. Fluorescence measurements of *eGFP* indicated that gene expression from the *tac* promoter was weaker in MEK700 medium. It was reported previously that strong expression of *hasA* led to HA with lower molecular weight in *S. zooepidermicus* (O'Regan *et al.* 1994). The combination of repression of cell growth and weaker expression of *hasA* improved molecular weight of the produced HA. The difference in molecular weight of the CGXII and the MEK700 HA was also detectable by the turbidimetric method with acetic albumin. The dependence of turbidity formation and polymerization degree of HA is known (Barker *et al*. 1963; Greiling 1963) and has to be taken account of when quantification of HA by a turbidimetric method is performed or a new turbidimetric assay is developed.

Investigations by microscopy revealed that *C. glutamicum* forms no detectable HA capsule. This might be due to the hydrophobic mycolic acids which are a special feature of the corynebacterial cell envelope (Lanéelle *et al.* 2013). The hydrophilic HA might be repelled from the cell surfaces. This could be an important economical factor, because no HA is retained by the cells and has to be extracted from them.

Altogether, *C. glutamicum* is a promising alternative host for the production of HA. It produced considerable amounts only by expressing the *hasA* gene without any further strain optimization. Balancing of the metabolic flux can rather be formulated as a key factor for high molecular weight HA production in *C. glutamicum* than the overexpression of precursor supplying genes, since this failed to enhance HA yield or molecular weight. Based on the results of this study, reasonable starting-points for optimization of HA production and increasing its molecular weight in *C. glutamicum* are balancing of *hasA* expression level and cell growth *e. g.* by control of oxygen supply or pH value. Lowering temperature enhanced molecular weight of HA produced by *S. zooepidermicus* (Armstrong and Johns 1997). This might also be an appropriate strategy for enhancing molecular weight of HA produced by *C. glutamicum*.

Acknowledgement

We thank Professor Dr. Benjamin Stark (Division of Biology, Department of Chemical and Biological Sciences, Illinois Institute of Technology, IIT Center, Chicago, IL 60616, USA) for pUC8:16. We also thank Silke Weber for technical assistance and the Landestiftung Baden Württemberg for financial support.

Conflict of interest

The authors declare no conflict of interest.

References

Abe, S., Takayama, K.I. and Kinoshita, S. (1967) Taxonomical studies on glutamic acid-producing bacteria. *J Gen Appl Microbiol* **13**, 279-301.

Armstrong, D.C. and Johns, M.R. (1997) Culture conditions affect the molecular weight properties of hyaluronic acid produced by *Streptococcus zooepidemicus*. *Appl Environ Microbiol* **63**, 2759-2764.

Balasz, E. A. (1979) Ultrapure hyaluronic acid and the use thereof. US patent no. 4141973A .

Barker, S. A., Bayyuk, I., Brimacombe, J. S., Hawkins, C. F. and Stacey, M (1963) Fingerprinting the hyaluronic acid component of normal and pathological synovial fluids. *Clin Chim Acta* **8**, 902-909.

Becker, J. and Wittmann, C. (2012) Bio-based production of chemicals, materials and fuels - *Corynebacterium glutamicum* as versatile cell factory. *Curr Opin Biotechnol* **23**, 631-640.

Beckers, G., Bendt, A.K., Krämer, R. and Burkovski, A. (2004) Molecular identification of the urea uptake system and transcriptional analysis of urea transporter- and urease-encoding genes in *Corynebacterium glutamicum*. *J Bacteriol* **186**, 7645-7652.

Bitter, T. and Muir, H.M. (1962) A modified uronic acid carbazole reaction. *Anal Biochem* **4**, 330-334.

Blombach, B., Riester, T., Wieschalka, S., Ziert, C., Youn, J.W., Wendisch, V.F. and Eikmanns, B.J. (2011) *Corynebacterium glutamicum* tailored for efficient isobutanol production. *Appl Environ Microbiol* **77**, 3300-3310.

Boeriu, C.G., Springer, J., Kooy, F.K., van den Broek, L.A.M. and Eggink, G. (2013) Production methods for hyaluronan. *Int J Carbohydr Chem* **2013**, article ID 624967, 14 Pages.

Buchinger, S., Stösser, J., Rehm, N., Hänßler, E., Hans, S., Bathe, B., Schomburg, D., Krämer, R. and Burkovski, A. (2009) A combination of metabolome and transcriptome analyses reveals new targets of the *Corynebacterium glutamicum* nitrogen regulator AmtR. *J Biotechnol* **140**, 68-74.

Burkovski, A. (2013) Cell envelope of corynebacteria: structure and influence on pathogenicity. *ISRN Microbiol* **2013**, article ID 935736, 11 pages.

Chassagnole, C., Diano, A., Létisse, F. and Lindley, N.D. (2003) Metabolic network analysis during fed-batch cultivation of *Corynebacterium glutamicum* for pantothenic acid production: first quantitative data and analysis of by-product formation. *J Biotechnol* **104**, 261-272.

Chien, L.J. and Lee, C.K. (2007) Enhanced hyaluronic acid production in *Bacillus subtilis* by coexpressing bacterial hemoglobin. *Biotechnol Prog* **23**, 1017-1022.

Cui, D., Jing, J., Boudou, T., Pignot-Paintrand, I., De Koker, S., De Geest, B.G., Picart, C. and Auzély-Velty, R. (2011) Hydrophobic shell loading of biopolyelectrolyte capsules. *Adv Mater* **23**, H200-H204.

DeAngelis, P.L., Jing, W., Drake, R.R. and Achyuthan, A.M. (1998) Identification and molecular cloning of a unique hyaluronan synthase from *Pasteurella multocida*. *J Biol Chem* **273**, 8454-8458.

Eggeling, L. and Bott, M. (2005) Handbook of *Corynebacterium glutamicum*. Boca Raton: CRC Press.

Eikmanns, B. (2005) Central Metabolism. In *Handbook of Corynebacterium glutamicum* ed. Eggeling, L. and Bott, M. pp. 241-276. Boca Raton: CRC Press.

Eikmanns, B.J. and Blombach, B. (2014) The pyruvate dehydrogenase complex of *Corynebacterium glutamicum*: An attractive target for metabolic engineering. *J Biotechnol* article in press, http://dx.doi.org/10.1016/j.jbiotec.2013.12.019.

Franklin, F.C., Bagdasarian, M., Bagdasarian, M.M. and Timmis, K.N. (1981) Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. *Proc Natl Acad Sci U S A* **78**, 7458-7462.

Greiling, H. (1963) Hyaluronic acid. In *Methods of Enzymatic Analysis* ed. Bergmeyer, H. U. pp. 87-92. New York: Academic Press.

Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D.C. and Jahn, D. (2005) JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res* **33**, W526-W531.

Hasegawa, S., Suda, M., Uematsu, K., Natsuma, Y., Hiraga, K., Jojima, T., Inui, M. and Yukawa, H. (2013) Engineering of *Corynebacterium glutamicum* for high-yield L-valine production under oxygen deprivation conditions. *Appl Environ Microbiol* **79**, 1250-1257.

Heider, S., Peters-Wendisch, P. and Wendisch, V. (2012) Carotenoid biosynthesis and overproduction in *Corynebacterium glutamicum*. *BMC Microbiol* **12**, 198.

Hubbard, C., McNamara, J.T., Azumaya, C., Patel, M.S. and Zimmer, J. (2012) The hyaluronan synthase catalyzes the synthesis and membrane translocation of hyaluronan. *J Mol Biol* **418**, 21-31.

Inui, M., Kawaguchi, H., Murakami, S., Vertès, A.A. and Yukawa, H. (2004) Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen-deprivation conditions. *J Mol Microbiol Biotechnol* **8**, 243-254.

Izawa, N., Hanamizu, T., Iizuka, R., Sone, T., Mizukoshi, H., Kimura, K. and Chiba, K. (2009) *Streptococcus thermophilus* produces exopolysaccharides including hyaluronic acid. *J Biosci Bioeng* **107**, 119-123.

Izawa, N., Serata, M., Sone, T., Omasa, T. and Ohtake, H. (2011) Hyaluronic acid production by recombinant *Streptococcus thermophilus*. *J Biosci Bioeng* **111**, 665-670.

Jia, Y., Zhu, J., Chen, X., Tang, D., Su, D., Yao, W. and Gao, X. (2013) Metabolic engineering of *Bacillus subtilis* for the efficient biosynthesis of uniform hyaluronic acid with controlled molecular weights. *Bioresour Technol* **132**, 427-431.

Keilhauer, C., Eggeling, L. and Sahm, H. (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol* **175**, 5595-5603.

Kim, J.H., Yoo, S.J., Oh, D.K., Kweon, Y.G., Park, D.W., Lee, C.H. and Gil, G.H. (1996) Selection of a *Streptococcus equi* mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. *Enzyme Microb Technol* **19**, 440-445.

Kogan, G., Šoltés, L., Stern, R. and Gemeiner, P. (2007) Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnol Lett* **29**, 17-25.

Lanéelle, M.A., Tropis, M. and Daffé, M. (2013) Current knowledge on mycolic acids in *Corynebacterium glutamicum* and their relevance for biotechnological processes. *Appl Microbiol Biotechnol* **97**, 9923-9930.

Letek, M., Fiuza, M., Ordóñez, E., Villadangos, A., Ramos, A., Mateos, L. and Gil, J. (2008) Cell growth and cell division in the rod-shaped actinomycete *Corynebacterium glutamicum*. *Antonie van Leeuwenhoek* **94**, 99-109.

Liu, L., Liu, Y., Li, J., Du, G. and Chen, J. (2011) Microbial production of hyaluronic acid: current state, challenges, and perspectives. *Microb Cell Fact* **10**, 99.

Mahony, J., Ainsworth, S., Stockdale, S. and van Sinderen, D. (2012) Phages of lactic acid bacteria: The role of genetics in understanding phage-host interactions and their co-evolutionary processes. *Virology* **434**, 143-150.

Mao, Z. and Chen, R.R. (2007) Recombinant synthesis of hyaluronan by *Agrobacterium* sp. *Biotechnol Prog* **23**, 1038-1042.

Mao, Z., Shin, H.D. and Chen, R. (2009) A recombinant *E. coli* bioprocess for hyaluronan synthesis. *Appl Microbiol Biotechnol* **84**, 63-69.

Moon, M.W., Park, S.Y., Choi, S.K. and Lee, J.K. (2007) The phosphotransferase system of *Corynebacterium glutamicum*: features of sugar transport and carbon regulation. *J Mol Microbiol Biotechnol* **12**, 43-50.

Necas, J., Bartosikova, L., Brauner, P. and Kolar, J. (2008) Hyaluronic acid (hyaluronan): a review. *Vet Med* **53**, 397-411.

Nešvera, J. and Pátek, M. (2011) Tools for genetic manipulations in *Corynebacterium glutamicum* and their applications. *Appl Microbiol Biotechnol* **90**, 1641-1654.

O'Regan, M., Martini, I., Crescenzi, F., De Luca, C. and Lansing, M. (1994) Molecular mechanisms and genetics of hyaluronan biosynthesis. *Int J Biol Macromol* **16**, 283-286.

Okino, S., Inui, M. and Yukawa, H. (2005) Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. *Appl Microbiol Biotechnol* **68**, 475-480.

Onaca, C., Kieninger, M., Engesser, K.H. and Altenbuchner, J. (2007) Degradation of alkyl methyl ketones by *Pseudomonas veronii* MEK700. *J Bacteriol* **189**, 3759-3767.

Prasad, S., Jayaraman, G. and Ramachandran, K. (2010) Hyaluronic acid production is enhanced by the additional co-expression of UDP-glucose pyrophosphorylase in *Lactococcus lactis*. *Appl Microbiol Biotechnol* **86**, 273-283.

Puskás, L. G., Inui, M., Yukawa, H. (2000) Structure of the urease operon of *Corynebacterium glutamicum*. *DNA Seq* **11**, 383-394.

Rados, D., Turner, D.L., Fonseca, L.L., Carvalho, A.L.c., Blombach, B., Eikmanns, B., Neves, A.R. and Santos, H. (2014) The effect of CO_2 on anaerobic succinate production by *Corynebacterium glutamicum*: carbon flux analysis by 13C-NMR. *Appl Environ Microbiol* **80**, 3015-3024.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. New York: Cold Spring Harbour Laboratory Press.

Sasaki, M., Jojima, T., Inui, M. and Yukawa, H. (2010) Xylitol production by recombinant *Corynebacterium glutamicum* under oxygen deprivation. *Appl Microbiol Biotechnol* **86**, 1057-1066.

Schröder, J. and Tauch, A. (2010) Transcriptional regulation of gene expression in *Corynebacterium glutamicum*: the role of global, master and local regulators in the modular and hierarchical gene regulatory network. *FEMS Microbiol Rev* **34**, 685-737.

Sheng, J.Z., Ling, P.X., Zhu, X.Q., Guo, X.P., Zhang, T.M., He, Y.L. and Wang, F.S. (2009) Use of induction promoters to regulate hyaluronan synthase and UDP-glucose-6-dehydrogenase of *Streptococcus zooepidemicus* expression in *Lactococcus lactis*: a case study of the regulation mechanism of hyaluronic acid polymer. *J Appl Microbiol* **107**, 136-144.

Smith, K., Cho, K.M. and Liao, J. (2010) Engineering *Corynebacterium glutamicum* for isobutanol production. *Appl Microbiol Biotechnol* **87**, 1045-1055.

Teramoto, H., Inui, M. and Yukawa, H. (2011) Transcriptional regulators of multiple genes involved in carbon metabolism in *Corynebacterium glutamicum*. *J Biotechnol* **154**, 114-125.

Unthan, S., Grünberger, A., van Ooyen, J., Gätgens, J., Heinrich, J., Paczia, N., Wiechert, W., Kohlheyer, D. and Noack, S. (2014) Beyond growth rate 0.6: What drives *Corynebacterium glutamicum* to higher growth rates in defined medium. *Biotechnol Bioeng* **111**, 359-371.

van der Rest, M.E., Lange, C. and Molenaar, D. (1999) A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* **52**, 541-545.

Werner, W., Rey, H.G. and Wielinger, H. (1970) Über die Eigenschaften eines neuen Chromogens für die Blutzuckerbestimmung nach der GOD/POD-Methode. *Fresenius Z Anal Chem* **252**, 224-228.

Widner, B., Behr, R., Von Dollen, S., Tang, M., Heu, T., Sloma, A., Sternberg, D., DeAngelis, P.L., Weigel, P.H. and Brown, S. (2005) Hyaluronic acid production in *Bacillus subtilis*. *Appl Environ Microbiol* **71**, 3747-3752.

Wieschalka, S., Blombach, B., Bott, M. and Eikmanns, B.J. (2013) Bio-based production of organic acids with *Corynebacterium glutamicum*. *Microb Biotechnol* **6**, 87-102.

Wittmann, C. and Heinzle, E. (2008) Metabolic network analysis and design in *Corynebacterium glutamicum*. In *Corynebacteria: Genomics and Molecular Biology* ed. Burkovski, A. pp. 79-112. Norfolk: Caister Academic Press.

Woolcock, J.B. (1974) The Capsule of *Streptococcus equi*. *J Gen Microbiol* **85**, 372-375.

Yamamoto, S., Sakai, M., Inui, M. and Yukawa, H. (2011) Diversity of metabolic shift in response to oxygen deprivation in *Corynebacterium glutamicum* and its close relatives. *Appl Microbiol Biotechnol* **90**, 1051-1061.

Yamamoto, S., Suda, M., Niimi, S., Inui, M. and Yukawa, H. (2013) Strain optimization for efficient isobutanol production using *Corynebacterium glutamicum* under oxygen deprivation. *Biotechnol Bioeng* **110**, 2938-2948.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Yu, H. and Stephanopoulos, G. (2008) Metabolic engineering of *Escherichia coli* for biosynthesis of hyaluronic acid. *Metab Eng* **10**, 24-32.

Figure Legends

Figure 1 Structure of the repeating disaccharide unit of hyaluronic acid composed of β-1,3 and β-1,4 linked D-glucuronic acid and *N*-acetylglucosamine.

Figure 2 Hyaluronic acid synthesis in *Streptococcus* sp. HasA: hyaluronic acid synthase, HasB: UDP-glucose 6-dehydrogenase, HasC: UTP-glucose 1-phosphate uridyltransferase, GlmU: bifunctional N-acetylglucosamine 1-phosphate uridyltransferase / glucosamine 1-phosphate acetyltransferase.

Figure 3 Construction of hyaluronic acid production shuttle vectors. The vectors contain pVWEx1 and pBR322 replication origins. The genes were integrated successively into pJOE7706.1 by using compatible *Spe*I/*Nhe*I restriction sites. Gene expression is controlled by the IPTG-inducible *tac* promoter. The cloned genes encode hyaluronic acid synthase (*hasA*), UDP-glucose 6-dehydrogenase (*hasB*), UTP-glucose 1-phosphate uridyltransferase (*hasC*), bifunctional N-acetylglucosamine 1-phosphate uridyltransferase / glucosamine 1-phosphate acetyltransferase (*glmU*), and *Vitreoscilla* haemoglobin (*vgb*).

Figure 4 Effects of the initial glucose concentration on the CDM and hyaluronic acid production of *C. glutamicum* pJH197.1 or wild type controls after 48 h of cultivation in MEK700 or CGXII minimal medium. (a) pJH197, MEK700. (b) pJH197, CGXII. (c) wild type, MEK700. (d) wild type, CGXII. (□) Hyaluronic acid contents of 1 ml uninduced culture supernatants. (■) Hyaluronic acid contents of 1 ml induced culture

supernatants. (○) CDM of uninduced cultures. (●) CDM of induced cultures. Data represent the means and standard deviations of three measurements.

Figure 5 Hyaluronic acid production and CDM of *C. glutamicum* strains in minimal medium. Cultures were first cultivated with an initial glucose concentration of (a) 0.5% (w/v) in MEK700 medium or (b) 1% (w/v) in CGXII medium and incubated for 48 h. Then, 4% (w/v) glucose were added and the cultures were further incubated for 72 h in (c) MEK700 medium or (d) CGXII medium. (□) Hyaluronic acid contents of 1 ml uninduced culture supernatants. (■) Hyaluronic acid contents of 1 ml induced culture supernatants. (○) CDM of uninduced cultures. (●) CDM of induced cultures. Data represent the means and standard deviations of three measurements.

Figure 6 Molecular weight analysis of hyaluronic acid produced by different *C. glutamicum* strains after 48 h of cultivation in (□) MEK700 minimal medium or (■) CGXII minimal medium. The dotted lines represent the elution volumes of dextran standard with indicated molecular weights. Data represent the means and standard deviations of three measurements.

Figure 7 Turbidity formation of HA of different molecular weights with acetic albumin (■) before or (□) after treatment with hyaluronidase. HA was produced by *C. glutamicum* pJH174.1 in MEK700 (>1.4 MDa) or CGXII (<270 kDa) minimal medium. The control HA originates from *Streptococcus equi* (1.5-1.8 MDa). The data represent the means and standard deviations of three measurements.

Figure 8 Microscopy of negative stained *C. glutamicum* cultures or culture supernatants (MEK700 minimal medium). (a) pJH174.1, uninduced culture. (b) pJH174.1, induced culture. (c) pJH174.1, uninduced culture supernatant. (d) pJH174.1, induced culture supernatant. (e) pJH174.1, uninduced culture supernatant + 50 mg l^{-1} hyaluronidase (1 h at 37°C). (f) pJH174.1, induced culture supernatant +

50 mg l^{-1} hyaluronidase (1 h at 37°C). (g) pJH182.1, uninduced culture. (h) pJH182.1, induced culture.

Tables

Table 1 Fluorescence of 0.01 OD_{600} *C. glutamicum* pJOE7706.1 24 h after induction

Minimal medium	uninduced	induced
CGXII*	24 ± 9	4,179 ± 51
MEK700†	35 ± 9	1,013 ± 56

*1% (w/v) Glucose, †0.5% (w/v) Glucose, data represent means and standard deviations of three measurements.

Table 2 pH values of culture supernatants after two-step cultivation of *C. glutamicum* wild type and hyaluronic acid producing strains

cultivation medium and time	wild type	HA-producers uninduced	HA-producers induced
MEK700* 48 h (0.5% glucose)	6.37 ± 0.02	6.41 ± 0.01	6.31 ± 0.04
MEK700* 120 h (0.5% + 4% glucose) ‡	4.34 ± 0.02	4.45 ± 0.02	4.55 ± 0.01
CGXII† 48 h (1% glucose)	8.76 ± 0.01	8.81 ± 0.01	8.70 ± 0.03
CGXII† 120 h (1% + 4% glucose) ‡	7.03 ± 0.01	6.94 ± 0.09	4.92 ± 0.07§

*initial pH value 7.3, †initial pH value 7.0, ‡additional glucose was added after 48 h incubation, §pH value of *C. glutamicum* pJH182.1 supernatant differed from the others (pH 6.78 ± 0.05) and is not included, data represent means and standard deviations of three measurements.

Table 3 Substrate-specific HA yields (mg g^{-1} consumed glucose) in MEK700 or CGXII minimal medium after two-step cultivation of *C. glutamicum* wild type and hyaluronic acid producing strains

	MEK700 48 h (0.5% glucose)		MEK700 120 h (0.5% + 4% glucose)		CGXII 48 h (1% glucose)		CGXII 120 h (1% + 4% glucose)	
	unind.*	ind.†	unind.	ind.	unind.	ind.	unind.	ind.
wild type	2.5 ± 0.5	2.6 ± 0.5	1.0 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	0.8 ± 0.0	0.5 ± 0.1	0.7 ± 0.1
pJH174.1	7.0 ± 1.2	57.3 ± 5.5	4.0 ± 0.1	17.0 ± 0.8	1.4 ± 0.6	32.9 ± 5.8	6.2 ± 0.5	22.9 ± 2.7
pJH181.3	6.7 ± 0.8	56.6 ± 3.1	3.8 ± 0.4	15.6 ± 1.1	1.5 ± 0.7	31.9 ± 4.8	6.2 ± 0.8	25.4 ± 4.1
pJH182.1	6.1 ± 1.1	25.3 ± 1.5	3.4 ± 0.4	7.5 ± 1.1	1.4 ± 0.5	22.0 ± 2.3	5.0 ± 0.7	14.4 ± 0.4
pJH183.2	6.9 ± 0.9	57.3 ± 4.8	3.8 ± 0.4	16.6 ± 0.5	1.5 ± 0.5	32.2 ± 5.9	6.6 ± 1.5	27.2 ± 5.0
pJH195.2	6.7 ± 1.0	34.6 ± 2.0	3.0 ± 0.5	14.4 ± 3.4	2.8 ± 0.2	39.3 ± 0.3	7.8 ± 0.8	25.6 ± 2.4
pJH196.2	6.5 ± 1.1	44.6 ± 2.3	3.4 ± 0.4	16.5 ± 0.5	2.6 ± 0.3	46.9 ± 1.1	7.3 ± 0.8	27.2 ± 1.1
pJH197.1	7.2 ± 0.9	41.6 ± 4.1	3.6 ± 0.4	13.1 ± 0.5	2.6 ± 0.4	50.4 ± 2.0	6.6 ± 0.4	24.8 ± 2.5

*uninduced, †induced with 1 mmol l^{-1} IPTG.