Understanding plasmid effect on hyaluronic acid molecular weight produced by *Streptococcus equi* subsp. *zooepidemicus*

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A B S T R A C T

Hyaluronic acid is a biopolymer with valuable applications in the pharmaceutical and cosmetic industries. *Streptococcus equi* subspecies *zooepidemicus* cells transformed with a nisin-inducible, empty plasmid control displayed higher molecular weight. This increase in molecular weight is independent of the nisin promoter or antibiotic resistance. Using 2D DIGE followed by mass spectrometry, we identified up-regulation of the last step in UDP-N-acetyl-glucosamine biosynthesis (GlmU) and down-regulation of the first step in peptidoglycan biosynthesis (MurA) as possible mechanism for the plasmid effect. Over-expression of GlmU to further increase activity had no effect on UDP-N-acetyl-glucosamine levels or molecular weight, while over-expression of MurA reduced UDP-N-acetyl-glucosamine levels and molecular weight. Global transcriptional analysis revealed that differential regulation of GlmU and MurA activity was not reflected in transcription levels. This results, suggest that regulation is at a translational or post-translational level. Differential expression of two clp proteases may explain this effect as well as the small but significant changes in transcription levels of nearly 300 genes.

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1. Introduction

Plasmids used for the over-expression of homologous and heterologous genes have profound effects on cellular metabolism (Bailey et al., 1986; Diaz-Ricci and Hernandez, 2000). Plasmid-carrying strains typically grow slower than their non-plasmid-carrying counterparts, a phenomenon attributed to the metabolic burden of carrying a plasmid. While naturally most pronounced in plasmids expressing high levels of proteins (Seo and Bailey, 1985), the effect is seen with plasmids alone even in the absence of selection (Diaz-Ricci et al., 1995). The metabolic burden – possibly in concert with more direct mechanisms – causes widespread changes in gene expression (Anthony et al., 2009; Birnbaum and Bailey, 1991; Mason and Bailey, 1989; Ow et al., 2006; Wang et al., 2006) and ultimately the metabolic phenotype defined by the metabolic flux distribution (Diaz-Ricci et al., 1995; Wang et al., 2006). While the exact mechanisms underlying the pleiotropic plasmid effects are not understood, it is a common practice to compare strains carrying plasmids over-expressing homologous and heterologous genes against "empty" or "mock transfection" vector controls rather than non-plasmid-carrying strains.

We recently demonstrated that the molecular weight (Mw) of the polysaccharide hyaluronic acid (HA) could be increased by over-expressing genes in the HA pathway (e.g., phosphoglucoisomerase (*pgi*)) in *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) (Chen et al., 2009). Similarly, Yu and Stephanopoulos (2008) demonstrated that *pgi* plays an important role in the production of high Mw HA in recombinant *Escherichia coli*. Surprisingly, the empty vector control also significantly increased the HA Mw to 2.5 MDa compared to the wild-type (WT) control at 1.8 MDa (Chen et al., 2009). In the presence of an empty plasmid *S. zooepidemcius* cells did not display a change in growth rate or HA yield. Thus, the change in Mw cannot be attributed to a metabolic burden effect, e.g., slowing down growth and freeing up upper glycolytic metabolites for HA biosynthesis.

In this study, the plasmid effect was explored to determine whether it was due to the inducer, nisin, or the selection with chloramphenicol. In addition, a proteomics approach using two-dimension differential in gel electrophoresis (DIGE) (Rathsam et al., 2005; Yan et al., 2002) was used to identify proteins potentially involved in the plasmid effect. The effect of one of the identified proteins – the enzyme UDP-N-acetylglucosamide 1-carboxivinyl transferase (*murA*) – was confirmed through over-expression.

2. Material and methods

2.1. Strains and culture conditions

All chemicals were purchased from Sigma Aldrich, unless otherwise specified.

S. zooepidemicus strain ATCC 35246 was obtained from the American Type Culture Collection (Rockville, MD, USA). The mucoid phenotype was preserved by culturing S. zooepidemicus on sheep blood agar and transferring it into a chemically defined medium modified from van de RIJN and Kessler (1980) by adding $20 \, g \, L^{-1}$ of glucose, $4.5 \, g \, L^{-1}$ of acetate and $50 \, mg L^{-1}$ of uridine. The medium contained $2.5 \, \mu g \, mL^{-1}$ chloramphenicol (Cm) or $1.5 \, \mu g \, mL^{-1}$ erythromycin (Em) with or without $20 \, ng \, mL^{-1}$ nisin.

A high Mw HA producer was obtained by over-expressing pgi as described previously (Chen et al., 2009). Growth experiments were conducted in a 2 L bioreactor (Applikon) at a working volume of 1.4 L and 37 °C. The reactor was agitated at 300 rpm and anaerobic conditions were maintained by top sparging with nitrogen during fermentation. The pH was controlled at 6.7 by addition of 5 M NaOH. All experiments were conducted at least in duplicate using an independent insertion for the mutant strain and the empty plasmid. Samples for protein and transcription analysis were harvested in exponential phase (OD_{530}=2–4).

2.2. Plasmid construction

Plasmid pNZ8148 was obtained from the Department of Biophysical Chemistry, NIZO (Mierau and Kleerebezem, 2005). Plasmid SynERM was synthesised using the backbone of the pNZ8148 vector with the replacement of the Cm^r resistance marker for the Em^r resistance marker (GeneArt AG) (Fig. 4, supplementary data). Plasmid pNZ8148Erm was generated by ligating the 1125 bp BglII–SalI fragment of SynERM to vector pNZ8148 cut with BglII and SalI using standard recombinant DNA techniques (Sambrook and Russell, 2001). The plasmid-free Lactococcus lactis (L. lactis) strain, MG1363 (Gasson, 1983), was selected on M17G agar supplemented with $5 \, \mu g \, mL^{-1}$ Cm or $3 \, \mu g \, mL^{-1}$ Em and employed as an intermediate host for all recombinant plasmids. The newly constructed plasmid was verified using Sanger sequencing. The recombinant plasmids were subsequently isolated from L. lactis MG1363 and electrotransformed into S. zooepidemicus.

The pgi strain was constructed as described by Chen et al. (2009). MurA genes were amplified from the S. zooepidemicus genome by PCR using the following primers:
murA1_F, AGTCCATGGCAGCTTATGGGATAAAATAATCATAG (NcoI);
murA1_R, TCTGCATGCTAACATATCTCCAACCACTAGTC (SphI);
murA2_F, AGTCTGCAGGTATCAGAAAGTTTTGAAAGTATGC (SpeI);
murA2_R, TGCACTAGTATGAAAGCCTTAAGTTCTTAATCCTC (PstI).

The conditions for PCR amplification, using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions with the primers, were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 20 s, 50 °C for 30 s, and 72 °C for 2 min 50 s, followed by 72 °C for 8 min. The size of the resultant product was confirmed by gel electrophoresis and gene inserts were cleaned using a QIAquick PCR Purification kit (Qiagen). The primers introduced restriction enzyme sites as required and the PCR fragments were cloned into pNZ8148 (Mierau and Kleerebezem, 2005) after digestion with the relevant restriction enzymes using standard recombinant DNA techniques (Sambrook and Russell, 2001). The full gene sequence and insertion sites were confirmed with Sanger sequencing. The recombinant plasmids were subsequently isolated from L. lactis MG1363 and electrotransformed into S. zooepidemicus (Table 1).

2.3. Electrantransformation of S. zooepidemicus

S. zooepidemicus cells were grown in M17 supplemented with $5 \, g \, L^{-1}$ glucose (M17G). After 12 h of incubation, cells were inoculated into 100 mL fresh M17G to 0.05 at OD 530 nm. The

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Lactococcus lactis MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	Gasson (1983)
Streptococcus equi subsp. zooepidemicus	$HA^+ \, Lac^+ \, Em^s$	ATCC 35246
Plasmids		
pNZ8148	Cm^r, nisin inducible expression vector, carrying the nisA promoter	Mierau and Kleerebezem, 2005
pNZ8148Erm BglII	Cm^r, Em^r; pNZ8148 derivative with an additional Em^r gene as a selectable marker	This work
pNZ8148Erm	Em^r, pNZ8148 derivative in which the Cm^r gene is replaced by the Em^r gene as a selectable marker	This work

Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance.

cells were further grown to about 0.6 at OD 530 nm. Prior to harvesting, $0.4 \, g \, L^{-1}$ of hyaluronidase was added followed by an additional incubation at 37 °C for another 30 min before centrifugation (Beckman Coulter, Avanti J-26 XPI: 5000G; 4 °C; 10 min). The supernatant was discarded, and the pellet was washed in 20 mL of electroporation buffer (0.5 M sucrose). After centrifugation, the pellet was washed again in 1 mL of the same buffer solution. Finally, the cells were resuspended in 250 μL of the buffer.

Electroporation was performed using a Bio-Rad Gene Pulser™ (Richmond, CA, USA) with pulse control. Ice-cold cuvettes of path length 0.2 cm containing 40 μL of washed cells and up to 4 μL purified plasmid DNA were used. Voltage was set at 3.0 kV (equivalent to $15 \, kV \, cm^{-1}$), resistance at $200 \, \Omega$ and capacitance at 25 μF. Immediately following pulse application, 1.0 ml of cold M17G broth was added to cells, which were then held on ice for 5 min prior to incubation at 37 °C for 2–3 h. Aliquots of electroporated cells (100 μL) were spread out on M17G agar plates supplemented with $2.5 \, \mu g \, mL^{-1}$ Cm or $1.5 \, \mu g \, mL^{-1}$ Em. All plates were incubated overnight at 37 °C.

2.4. Analytical techniques

Biomass and fermentation products (including HA, lactate, acetate, formate, glucose and ethanol) were measured by collecting cell broth samples hourly as described by Chen et al. (2009). Intracellular metabolites were measured as described elsewhere (Marcellin et al., 2009b). Mw determination was performed using a Lauda Viscosity measuring system as described elsewhere (Chen et al., 2009). Enzyme assays were performed as described previously (Chen et al., 2009).

2.5. 2 Dimencion Differential gel electrophoresis (DIGE)

Proteomics was used to investigate the enzymatic interactions and regulation that affect Mw control in bacterial HA fermentation. 2D DIGE was used to separate proteins and to quantify the level of expression in (1) WT, (2) strain harbouring pNZ8148 and (3) the high Mw producer over-expressing pgi.

Protein samples extracted from triplicate cultures were labelled with CyDye using the experimental procedure illustrated in Fig. 5 (Supplementary data). A mixture of all samples was used as an internal standard and was labelled with Cy2. Iso-electric focusing was performed using 24 cm IPG strips according to