Hyaluronic Acid Production in *Bacillus subtilis*

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The *hasA* gene from *Streptococcus equisimilis*, which encodes the enzyme hyaluronan synthase, has been expressed in *Bacillus subtilis*, resulting in the production of hyaluronic acid (HA) in the 1-MDa range. Artificial operons were assembled and tested, all of which contain the *hasA* gene along with one or more genes encoding enzymes involved in the synthesis of the UDP-precursor sugars that are required for HA synthesis. It was determined that the production of UDP-glucuronic acid is limiting in *B. subtilis* and that overexpressing the *hasA* gene along with the endogenous *tuaD* gene is sufficient for high-level production of HA. In addition, the *B. subtilis*-derived material was shown to be secreted and of high quality, comparable to commercially available sources of HA.

Hyaluronic acid (also referred to as HA or hyaluronan) is a linear, unbranched polysaccharide made of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid and can reach chain lengths of up to 20,000 disaccharide units or higher (8 MDa) (44). HA exists in nature as a hydrated gel and is ubiquitous in human and animal tissues (37). This macromolecule influences cell behavior and has significant structural, rheological, physiological, and biological functions in the body (21, 24, 39). Its distinctive viscoelastic properties, coupled with its lack of immunogenicity or toxicity, have led to a wide range of applications in the cosmetic and pharmaceutical industries, including skin moisturizers, osteoarthritis treatment, ophthalmic surgery, adhesion prevention after abdominal surgery, and wound healing (12, 47). At present the worldwide market for HA is estimated at over \$1 billion. HA is obtained commercially from rooster combs and certain attenuated strains of group C *Streptococcus* which synthesize this compound naturally as part of their outer capsule (3, 15). However, these are less-than-ideal sources. All rooster comb-based HA products carry warnings directed to those who are allergic to avian products, and at least one has been reported to cause inflammatory reactions upon injection (30), while streptococci can be difficult or expensive to ferment, are challenging to genetically manipulate, and have the potential to produce exotoxins. Thus, it would be advantageous to develop an alternative source of HA that avoids these serious pitfalls.

Bacillus species have long been established as industrial workhorses for the production of products ranging from hydrolytic enzymes, such as proteases and alpha-amylases, to specialty chemicals, such as amino acids and vitamins (11, 13, 28, 31). These organisms are capable of secreting copious amounts of product, indicative of their highly developed biosynthetic capacity, and are very economical to grow in industrial fermentors. *Bacillus subtilis* is free of exotoxins and endo-

toxins and, consequently, many products produced in this organism have been awarded a GRAS (generally recognized as safe) designation. In addition, *B. subtilis* does not produce, nor does the genome sequence encode, a hyaluronidase which could degrade HA. Finally, *B. subtilis* is one of the most-well-characterized gram-positive microorganisms, its genome has been sequenced (23), and there are wide arrays of tools available for genetic manipulation. Thus, this bacterium offers several advantages as a possible expression host for HA production.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains One Shot and TOP10 (Invitrogen) were used for cloning purposes. *B. subtilis* strains 168Δ4 and A164Δ5 were used for strain constructions. Strain A168Δ4 is essentially *B. subtilis* 168 (BGSC 1A1; Bacillus Genetic Stock Center, Columbus, OH) which has deletions in the *amyE*, *spoIIAC*, *aprE*, and *nprE* genes (the same as those described for A164Δ5; the latter strain is derived from the ATCC 6051a background and has an extra deletion in the *srfC* gene) (33). *B. subtilis* cells were made competent by the method of Anagnostopoulos and Spizizen (2). Cells were grown either on LB or tryptose blood agar base (Difco) plates supplemented with chloramphenicol at 5 μg/ml, neomycin at 5 μg/ml, or ampicillin at 100 μg/ml, as appropriate. To score the mucoid colony phenotype, *B. subtilis* cells were also grown on minimal media plates (14) without antibiotics.

Plasmid vectors. Plasmid pCR2.1 (Invitrogen) was used for cloning PCR fragments. Plasmids pNNBT20 (see Fig. 2A), referred to elsewhere as pDG268Δneo-long cryIIIAstab/SAV (45), and pNNBT21 (see Fig. 2B), referred to elsewhere as pDG268Δneo-short “consensus” *amyQ*/SAV (45), were used for constructing artificial operons and introducing them into the *B. subtilis* genome, respectively.

PCR cloning of genes. The following genes were PCR amplified and cloned into plasmid pCR2.1: the *tuaD*, *gtaB*, and *gcaD* genes from *B. subtilis* 168Δ4 and the *hasA* gene from *Streptococcus equisimilis* (22). The strong ribosome binding site from the *Bacillus clausii* *aprL* gene (5) was utilized as the translation initiation signal for *hasA*; the other genes utilized their endogenous ribosome binding sites. Each ribosome binding site/gene fragment was engineered via PCR to be flanked by unique restriction sites: *SacI*/*hasA*/*KpnI*/*HpaI*, *KpnI*/*tuaD*/*XbaI*/*HpaI*, *XbaI*/*gtaB*/*BamHI*/*HpaI*, and *XbaI*/*gcaD*/*HpaI*. Details on the assembly of the expression cassettes have been published elsewhere (34).

Construction of *B. subtilis* strains. The genes described in the previous section were used to construct four expression cassettes which were introduced into *B. subtilis* for analysis. The approach was to first assemble the gene combinations in the promoterless plasmid pNNBT20; a promoter for transcribing these genes was avoided mainly for stability concerns in *E. coli*. The next step was to transcriptionally fuse each of these gene combinations to the relatively strong “short

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consensus" *amyQ* promoter (46) and introduce each completed cassette into the *amyE* locus on the chromosome of *B. subtilis*. The presence and integrity of the expression cassettes was confirmed by PCR analysis. The last step was to transfer all four cassettes into the higher-producing *B. subtilis* strain A164Δ5. This was accomplished by isolating chromosomal DNA (29) from the four 168Δ4 strains harboring these cassettes and using the DNA to transform A164Δ5 (a poorly transformable strain) to chloramphenicol resistance. The resulting strains were designated RB161, RB163, RB184, and RB182 (see Fig. 2, below). The first three strains exhibited a mucoid phenotype on minimal media plates (14). Details on these strain constructions are published elsewhere (34).

Fermentations. The host strain, *B. subtilis* A164Δ5, is capable of growth on a mineral salts medium supplemented with only carbohydrate and biotin. Because of its simplicity, a minimal medium was used with sucrose as the carbohydrate. Strains were grown in 3-liter fermentors. The pH was adjusted to 6.8 to 7.0 with ammonia before inoculation and controlled thereafter at 7.0 ± 0.2 with ammonia and H_3PO_4 . The temperature was maintained at 37°C. Agitation was at a maximum of 1,300 rpm using two six-bladed Rushton impellers of 6-cm diameter in a 3-liter tank with an initial volume of 1.5 liters. The aeration had a maximum of 1.5 VVM (liters air/liters fermentor broth/minute).

For feed, a simple sucrose solution was used. Feed started at about 4 h after inoculation, at a time when dissolved oxygen was still being driven down (i.e., before sucrose depletion). The feed rate was ramped linearly from 0 to ≈ 6 g sucrose/liter-h over a 7-h time span.

Viscosity was noticeable by about 10 h and by 24 h viscosity was very high, causing the dissolved oxygen to plummet. Cell mass development reached a near maximum (12 to 15 g/liter) by 20 h. Cell removal was done by diluting 1 part culture with 3 parts water, mixing well, and centrifuging at about $30,000 \times g$, yielding a clear supernatant and a cell pellet that could be washed and dried.

Hyaluronan analyses. HA titers were routinely estimated by the carbazole assay (4) after first precipitating HA from medium samples with cetyl pyridinium chloride (1.7%, wt/vol) and then redissolving in 1.0 M NaCl. The assay detects the glucuronic acid released after the sample has been hydrolyzed with H_2SO_4 . This assay is not highly specific (compounds such as sucrose are cross-reactive), and so it was necessary to determine the background reading after removal of HA by precipitation. The HA titer is assumed to be 2.05 times the glucuronic acid titer.

Selected samples were analyzed for HA content using the interaction of HA with a specific biotinylated HA binding protein using the HA quantitative test kit from Corgenix, Westminster, CO. This assay currently provides the most sensitive and specific way to quantify HA and also serves to validate the usefulness of the carbazole assay, since in general the agreement between the two assays was good ($\pm 15\%$). Multiple dilutions of medium samples were prepared and assayed in triplicate along with HA standards according to the manufacturer's instructions.

Gel permeation chromatography in combination with multiangle laser light scattering was used to determine weight-average molecular mass (48). The column was a model PL Aquagel-OH 60 (15 μ m; 300×7.5 mm) from Polymer Laboratories, Inc. (Amherst, MA). The column eluate first flowed through an Optilab DSP refractometer to estimate HA concentrations and then through a 16-detector Dawn DSP laser photometer to measure light scattering (both instruments were from Wyatt Technology Corp., Santa Barbara, CA).

RESULTS

Expression of HA via artificial operons in *B. subtilis*. HA is synthesized by the polymerization of the monosaccharides from the two nucleotide sugars UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc). The enzyme that catalyzes this reaction is HA synthase or HAS. To date, genes encoding HASs from prokaryotes, eukaryotes, and even an algal virus have been cloned and sequenced (8, 9, 10, 18, 22, 32, 36, 42). Only a few bacterial species are known to produce HA, namely the group A and group C streptococci (gram positive) and *Pasteurella multocida* (gram negative) (6, 19, 25). Since *B. subtilis* is also a gram-positive bacterium, it seemed logical to exploit the wealth of information which is available for the streptococci concerning HA production.

The pathway for HA biosynthesis in the group A and group C streptococci has been determined (26, 27), and it can be readily adapted to optimize HA production in *B. subtilis* as well

(Fig. 1); the only enzyme *B. subtilis* lacks is the hyaluronan synthase, which is encoded by the *hasA* gene. Starting from glucose as a carbon source, two parallel metabolic branches can form which eventually, through multiple sugar intermediates, culminate in the synthesis of the two nucleotide sugar substrates, UDP-GlcUA and UDP-GlcNAc. It is clear that HA biosynthesis is an expensive proposition for the cell, both in terms of carbon and energy consumption. For every mole of HA disaccharide unit produced, 2 mol of glucose, 5 mol of nucleoside triphosphates (3 as ATP and 2 as UTP), and 1 mol of acetyl-coenzyme A are consumed. If large amounts of HA are produced, this could pose a substantial metabolic burden on the cell. In addition, several of the sugars included in this pathway are also required for important cellular functions. The nucleotide sugars UDP-Glc and UDP-GlcNAc are both required for cell wall biosynthesis. The phosphosugars Glc-6-P and Fru-6-P funnel directly into the pentose phosphate and glycolytic pathways, respectively, both of which are essential for cell growth. In order for the cell to synthesize large quantities of HA, it is imperative that these various sugar metabolites be maintained at adequate levels to sustain cell growth.

The streptococcal cell has evolved a dedicated expression system to overcome this predicament. The *hasA* gene has been incorporated as the first gene of an operon along with one or more genes (hereafter referred to as "precursor genes") which encode enzymes that are involved in the synthesis of some of the aforementioned precursor sugars. To date, the HAS operons from four streptococcal species have been cloned and sequenced. The operon from *Streptococcus pyogenes* contains three genes: the first, *hasA*, encodes hyaluronan synthase; the second, *hasB*, encodes UDP-Glc dehydrogenase; and the third, *hasC*, encodes UDP-Glc pyrophosphorylase (7). The operon from *Streptococcus uberis* is somewhat different in that the *hasC* gene is not present as part of the operon but is located elsewhere on the chromosome (41). The operons from *S. equisimilis* and *Streptococcus zooepidemicus* subsp. *equi* contain the *hasA*, *hasB*, and *hasC* genes as well as a fourth gene, *hasD*, which encodes UDP-GlcNAc pyrophosphorylase (34). In addition, the latter strain contains a fifth gene, *hasE*, which encodes phosphoglucoisomerase (Lars Nielsen, Chemical Engineering Department, University of Queensland, Brisbane, Australia, personal communication). Thus, these streptococcal species have evolved expression systems via operons for ensuring that adequate levels of various precursor sugars are available to support both HA biosynthesis and cell growth.

In order to produce HA in *B. subtilis*, it seemed logical to take a cue from nature and mimic this approach. This could be accomplished in a few different ways. A *has* operon from a group A or C *Streptococcus* could simply be introduced into *B. subtilis*. Alternatively, artificial operons could be constructed which are comprised of *B. subtilis* genes as well. The latter approach is appealing, since endogenous genes are frequently expressed more efficiently in their native host. *B. subtilis* has homologues to the *hasB*, *hasC*, and *hasD* genes, and they are designated *tuaD*, *gtaB*, and *gcaD*, respectively. Artificial operons were therefore constructed which contained the *hasA* gene from streptococcus along with one or more of these latter *B. subtilis*-derived genes (Fig. 2C). For example, strain RB161 contains an artificial operon that mimics the *S. pyogenes hasA*-

FIG. 1. Proposed biosynthetic pathway for HA production in recombinant *B. subtilis* strains. This pathway is based on the reported HA biosynthetic pathway in group A and C streptococci (26, 27), homology searches against the published *B. subtilis* genome sequence, and the *B. subtilis* metabolic pathways described in the Kyoto Encyclopedia of Genes and Genomes (<http://bioinformatics.weizmann.ac.il:3456/kegg/kegg.html>). Gene designations are highlighted in boldface. The *B. subtilis* homologs of streptococcal *has* genes are in parentheses.

hasB-*hasC* locus. Likewise, the operon in strain RB184 mimics the *S. uberis* *hasA*-*hasB* locus.

Experiments have focused on HA production using the *hasA* gene from *S. equisimilis*, since the hyaluronan synthase from this organism is especially interesting due to its higher intrinsic polymerization rate (22). The promoter for driving the expression of these operons is a mutated version of the *amyQ* promoter from *Bacillus amyloliquefaciens* and is a relatively strong promoter in *B. subtilis* (46). The *hasA* gene in each construct utilizes a strong ribosome binding site derived from the *aprL* gene from *B. clausii* (5). The *B. subtilis* genes, *tuaD*, *gtaB*, and *gcaD*, utilize their own ribosome binding sites, each of which was engineered to be located approximately 50 bases downstream of the preceding gene in the operon. A strong transcription terminator from the *aprL* gene of *B. clausii* is located at the 3' end of each operon. All of the expression cassettes were introduced into the chromosome of *B. subtilis* A164Δ5 (33) in the *amyE* locus. This particular strain was chosen due to its superior growth characteristics and high yield of secreted protein products in industrial fermentors.

During the construction of these strains it became readily apparent which constructs were proficient at producing HA as evidenced by a mucoid colony morphology on plates. This

morphology became even more noticeable when the strains were grown on minimal medium plates with glucose as a sole carbon source (Fig. 3).

Production of HA in lab-scale fermentors. One of the strains which produced a pronounced mucoid colony morphology on a plate, RB161, was selected and grown in a 3 liter lab-scale fermentor on a minimal medium with sucrose as the carbon source (Fig. 4). Samples were collected at various times during the fermentation and measured for HA titer, viscosity, and dissolved oxygen. Maximum production was reached at approximately 25 h into the fermentation. The amount of HA produced was in the multigram per liter range and compared favorably to published yields obtained from streptococcal fermentations (20). Due to the viscoelastic properties of HA, the fermentation became very viscous at about 15 h into the fermentation and concomitantly the dissolved oxygen concentration plummeted. The viscosity reached a peak at 25 h and slowly decreased throughout the remainder of the fermentation, even though the concentration of HA remained the same or slightly increased. The reason for this phenomenon is unknown. The production of a hyaluronidase by the *Bacillus* host seems unlikely based on the observation that the genome se-

FIG. 5. Effect of precursor gene overexpression on HA productivity in *B. subtilis*. The HA content of a medium sample, at the indicated times, was determined. The genes in the artificial *has* operons of the indicated strains are in parentheses.

quence for the *B. subtilis* 168 type strain does not appear to encode homologs to any of the known hyaluronidase enzymes.

The HA produced in the fermentation was analyzed by gel permeation chromatography in combination with multiangle laser light scattering to determine molecular mass and polydispersity. The *B. subtilis*-derived material consistently measured in the 1.1- to 1.2-MDa range with a polydispersity index of 1.5 (typically expressed as the ratio of weight-average molecular mass/number-average molecular mass, or M_w/M_n for a given polymer), comparable to what has been reported for commercially available sources (1).

UDP-glucuronic acid is limiting for HA synthesis in *B. subtilis*. Significant amounts of HA are produced by strain RB161. Besides the *hasA* gene, this strain has been engineered to overexpress the *tuaD* and *gtaB* genes, whose enzyme products are involved in the synthesis of UDP-GlcUA and UDP-Glc, respectively. In order to determine if either of these UDP-sugars is limiting for HA production, strains RB182 and RB184 were constructed; these strains overexpress *hasA* alone or *hasA* and *tuaD*, respectively. Both strains were grown in fermentors and analyzed for HA production (Fig. 5). Yields from RB182 were negligible, whereas yields from RB184 were comparable to strain RB161, indicating that the level of UDP-GlcUA is limiting in the native *B. subtilis* background due to insufficient levels of UDP-Glc dehydrogenase. This observation is actually expected in that the native *tuaD* gene is reported to be repressed by phosphate, which is in excess during these fermentations (35).

Overexpression of the precursor genes *hasB* and *hasC* is expected to increase the levels of UDP-GlcUA in the cell. We postulated that increasing the levels of the other sugar substrate, UDP-GlcNAc, could also have a positive impact on HA production. The *hasA* operon from *S. equisimilis* does contain a fourth gene, *hasD*, which encodes UDP-GlcNAc pyrophosphorylase. This bifunctional enzyme catalyzes the last two steps in the synthesis of UDP-GlcNAc. To test whether overproducing this particular enzyme could further boost HA yields, strain RB163 was constructed. This strain overexpresses *gcaD*, the *B. subtilis* homolog to *hasD*, in addition to *hasA* and *tuaD*. Fermentation results from this strain showed that HA yields were essentially equal to those obtained from RB161 and RB184 (Fig. 5). Similar results were obtained from a *B. subtilis* strain expressing the *S. equisimilis* operon, *hasA/hasB/hasC/hasD*

(data not shown). These results could indicate that HA production by RB161 and RB184 is not limited by UDP-GlcNAc or perhaps, if it is, the limiting step is earlier in the biosynthetic pathway than the step catalyzed by UDP-GlcNAc pyrophosphorylase, which leads to this precursor sugar.

DISCUSSION

B. subtilis has proven to be a superior expression host for producing HA based on several criteria. The yield and quality of the product are very good, both with regards to molecular mass and polydispersity. In addition, the *B. subtilis*-derived HA is exotoxin free, which can be problematic in certain streptococcal strains. The HA is secreted into the surrounding medium and is not cell associated, which should greatly simplify the recovery process and facilitate downstream processing. The production economics are further enhanced due to the ability of *B. subtilis* to grow on minimal media, in contrast to the streptococcus groups A and C, which are fastidious organisms and require more expensive complex media for growth.

In *B. subtilis*, we have shown that the levels of UDP-GlcUA are limiting with regards to high-level HA production, at least when grown in the presence of excess phosphate. This was shown conclusively by comparing the *hasA*- and the *hasA/tuaD*-overexpressing strains. It is interesting that overexpressing *hasA* alone does not seem to hinder cell growth, i.e., the hyaluronan synthase is not titrating away sugars that are required for cell growth, at least not to dangerously low levels. This is in contrast to *E. coli*, where overexpression of *hasA* by itself severely compromises the cell's ability to grow (10); this effect can be avoided if a strain lacking the *ugd* gene (a *tuaD* homolog) is utilized. Evidently, in *B. subtilis* this is not a problem, as evidenced by the *hasA/tuaD* overexpressor, in which high-level production of HA and cell growth are supported simultaneously. Clearly, *B. subtilis* is inherently more metabolically robust than *E. coli*, at least with regards to HA production.

In group A and group C *Streptococcus*, it has been shown that the hyaluronan synthase enzyme is an integral membrane protein (16). It has been proposed that this enzyme, in association with phospholipids, forms a pore for transporting the growing HA chain through the membrane (38). The fact that HA is produced very efficiently in *B. subtilis* indicates that the streptococcal hyaluronan synthase enzyme is, in fact, folding and assembling properly within the membrane; no other exogenous components seem to be required. This is in contrast to the HA-producing gram-negative bacterium *Pasteurella multocida*, which appears to require a multicomponent system, in addition to hyaluronan synthase, for transporting the growing HA chain to the outside of the cell (40). Certainly, choosing a *hasA* gene from a gram-positive *Streptococcus* was a logical choice for *B. subtilis*.

Considering the ease with which *B. subtilis* can be manipulated, this expression system could lend itself as an ideal model system for dissecting the complex enzymology of hyaluronan synthase. Even though it is a small protein, this enzyme must perform multiple functions in order to polymerize the two sugars and transport the growing HA polymer to the outside of the cell; no less than seven distinct functions have been proposed (43). Structure-function studies have yielded valuable bits of information concerning HAS topology and organization

within the membrane, but so far they have been limited to low numbers of site-directed mutants and measures of enzyme function have been limited to in vitro assays (17). It would be advantageous to have a system whereby large numbers of engineered HAS variants could easily be generated and screened in vivo for activity. Technically, this would be extremely challenging with a streptococcus-based system, due to the difficulties and inefficiencies in genetically manipulating this organism. However, a *B. subtilis*-based expression system should be very amenable to this sort of analysis. Ultimately, a more detailed understanding of HA synthesis could lead to custom-tailored products, such as targeted molecular weight ranges, some of which will undoubtedly result in the development of new and exciting applications for this versatile product in the future.

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