

Expression of Low Endotoxin 3-*O*-Sulfotransferase in *Bacillus subtilis* and *Bacillus megaterium*

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Abstract A key enzyme for the biosynthesis and bioengineering of heparin, 3-*O*-sulfotransferase-1 (3-OST-1), was expressed and purified in Gram-positive *Bacillus subtilis* and *Bacillus megaterium*. Western blotting, protein sequence analysis, and enzyme activity measurement confirmed the expression. The enzymatic activity of 3-OST-1 expressed in *Bacillus* species were found to be similar to those found when expressed in *Escherichia coli*. The endotoxin level in 3-OST-1 from *B. subtilis* and *B. megaterium* were

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10^4 – 10^5 -fold lower than that of the *E. coli*-expressed 3-OST-1, which makes the *Bacillus* expression system of particular interest for the generation of pharmaceutical grade raw heparin from nonanimal sources.

Keywords 3-*O*-Sulfotransferase · Bioengineered heparin · Endotoxin free · *Bacillus subtilis* · *Bacillus megaterium* · Nonanimal sources

Introduction

Heparin is the most widely used anticoagulant drug in modern medicine. Approximately 300,000 doses/day are used in the USA, and greater than 100 tons of heparin is used annually worldwide, with a market value of ~\$7 billion [1]. Heparin is a highly sulfated polysaccharide found covalently attached to the core protein serglycin and stored in intracellular granules of mast cells that are found in the intestines and lungs of many animals [1]. A health crisis in 2008, involving the adulteration of heparin produced from hogs in China, led to the death of as many as 100 Americans and resulted in a demand for heparin from nonanimal sources [2]. In 2007, our laboratories chemoenzymatically synthesized a small amount of bioengineered heparin from an *Escherichia coli*-derived polysaccharide [3]. The process begins with an *E. coli* fermentation to prepare polysaccharide, chemical de-*N*-acetylation, and *N*-sulfonation, and then the use of recombinant *O*-sulfotransferases and C5-epimerase results in a bioengineered heparin that closely resembles the chemical and biological properties of pharmaceutical heparin. In our previous research, all the biosynthetic enzymes were expressed in *E. coli* [4], which releases endotoxin contaminating the bioengineered heparin product. The endotoxin resulting from the recombinant *E. coli* enzyme expression complicates the purification procedure, potentially increasing the production cost. We selected the Gram-positive bacteria *Bacillus subtilis* and *Bacillus megaterium* as hosts to express murine 3-*O*-sulfotransferase-1 (3-OST-1), a critical enzyme for biosynthesis of bioengineered heparin [1], to reduce endotoxin levels.

B. subtilis is an attractive industrial microorganism, which is marked with high growth rate, generally regarded as safe status, and endotoxin free and has the capacity to secrete proteins into the medium [5]. It is becoming an occasional alternative host to *E. coli* to produce recombinant proteins in the biopharmaceutical industry and as an industrial host for the expression of enzymes. With the recent advances in the *B. subtilis* expression system, the major shortcomings for using *B. subtilis* to express heterologous proteins, such as a lack of suitable expression vectors, plasmid instability, and presence of proteases, have been improved significantly [5]. However, production of high-value eukaryotic proteins for pharmaceutical applications remains a major challenge in *B. subtilis*. One problem with the production of eukaryotic proteins is the absence of expression or relatively low expression efficiency [6]. Overproduction of recombinant proteins in *B. subtilis* is a complex process requiring the design of improved production strains and a comprehensive understanding of the cellular physiology and protein expression mechanisms.

Similarly, *B. megaterium* is also becoming a widely used cellular factory for the production of heterologous proteins. Shuttle vectors have become available that allow for cloning in *E. coli* and expression in *B. megaterium*. This bacterium has been shown to produce many industrial and commercially important products including antimicrobial synthetic proteins and vitamins [7]. However, protein expression is often higher in *B. megaterium* than in *B. subtilis*, and *B. megaterium* lacks alkaline proteases found in *B. subtilis* and also exhibits good plasmid stability [7].

Table 1 Primers used for cloning of 3OST1opt into pHT01 and pP_{T7}

Primer	Sequence (5'–3')	Amplified
P14for	GCTCTAGAAATGCATCATCATCATCATCA	XbaI-6×His-3OST1opt-SmaI
P14rev	ACCCGGGTCAGTGCCAATCA	
P15for	GGCCATACTAGTATGCATCATCATCATCATCA	SpeI-6×His-3OST1opt-SmaI
P15rev	GGCCATCCCGGGTCAGTGCCAATCA	

Italics denotes restriction site

Materials and Methods

Bacterial Strains, Plasmids, and Genes

The catalytic domain of *3-ost-1* gene (GenBank accession no. AF019385.1; amino acids sequence from G48 to H311) [8] was codon optimized and synthesized by Integrated Device Technology, Inc. (USA) and cloned into the pIDTSMART plasmid with a 5' overhang containing an *Xba*I site, a 3' overhang containing an *Sma*I site, and a 6× His-tag fused at the N-terminus of expressed protein. The pIDTSMART-3-OST-1 plasmid was double digested using *Xba*I and *Sma*I restriction enzymes (Fermentas, USA), and the digested *3-ost-1* gene was ligated into the *E. coli*-*B. subtilis* shuttle vector pHT01 (MoBiTec, Germany). For sequencing, *E. coli* Top10 strain (Invitrogen, USA) was transformed with pHT01-3-OST. After sequence confirmation, *B. subtilis* strain 1012wt (MoBiTec, Germany) was then transformed with pHT01-3-OST-1.

The *3-ost-1* gene was then amplified from the pHT01-3-OST-1 vector, including *Spe*I and *Sma*I restriction sites at the 5' and 3' ends, respectively. After digestion and purification, the digested *3-ost-1* gene was ligated into the *E. coli*-*B. megaterium* shuttle vector pP_{T7} (MoBiTec, Germany). For sequencing, *E. coli* Top10 strain (Invitrogen, USA) was transformed with the pP_{T7}-3-OST-1 plasmid, and after sequence confirmation, *B. megaterium* strain MS941 (MoBiTec, Germany) was transformed with pP_{T7}-3-OST-1. For expression under the control of the T7 promoter, the strain was also transformed with the pT7-RNA polymerase (pT7-RNAP) (MoBiTec, Germany) plasmid encoding the T7 RNA polymerase. Primers used for cloning into both pHT01 and pP_{T7} are described in Table 1.

Transformations into *Bacillus* Species and Protein Production

The *B. subtilis* strain 1012wt was transformed with pHT01-3-OST-1 with a previously described method [5], and transformants were selected for with chloramphenicol (10 µg/mL) on lysogeny broth (LB) agar.

B. megaterium strain MS941 was transformed with pP_{T7}-3-OST-1 and pP_{T7}-RNAP via a protoplast-mediated method [9]. Briefly, *B. megaterium* cells were grown to late log phase, centrifuged, and incubated with lysozyme to create *B. megaterium* protoplasts, which were then incubated with plasmid DNA for transformation and plated on LB containing chloramphenicol (10 µg/mL) for pP_{T7}-RNAP transformants and, additionally, tetracycline (10 µg/mL) for co-transformants (cells transformed with both pP_{T7}-RNAP and pP_{T7}-3-OST-1).

Single transformants were grown overnight in LB media (10 g/L NaCl, 10 g/L casein tryptone, 5 g/L yeast extract) supplemented with appropriate antibiotics at 37 °C and 220 rpm. The cultures were then inoculated into 100 mL fresh LB containing appropriate antibiotic at a 1:50 ratio. 3-OST-1 expression was induced at OD_{600 nm} 0.7–0.8 using either

1 mM IPTG for *B. subtilis* or 0.5 % (w/v) xylose for *B. megaterium*. After 18 h of induction at room temperature, cells were harvested by centrifugation, and the cell pellet was resuspended in lysis buffer (25 mM Tris–HCl, 500 mM NaCl) and sonicated to obtain cell lysate. 3-OST-1 production was monitored with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and verified by Western blotting with 3-OST-1 antibodies (Santa Cruz Biotechnology, Inc., USA).

3-OST-1 Protein Sequence Analysis by Liquid Chromatography–Tandem Mass Spectrometry

The overexpressed protein band from *B. subtilis* was in-gel digested, and its protein sequence was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) to further confirm its identity as 3-OST-1. Briefly, the band was excised from the SDS-PAGE gel, cut into small pieces, digested with trypsin, and extracted with buffer several times into aqueous acetonitrile (50 %v/v) containing formic acid (5 %v/v). The extract was concentrated using a vacuum concentrator and analyzed by LC-MS/MS.

Purification of 6× His-Tagged 3-OST-1

Purifying 6× His-tagged 3-OST-1 proved problematic under native conditions using a nickel column whether the His-tag is cloned onto the N-terminus or the C-terminus of the protein, either because most of the protein is present in inclusion bodies or because the His-tag is folded in the protein and unable to bind the column. However, the protein was purified from the cell lysate from both *B. subtilis* and *B. megaterium* using a nickel column under denaturing conditions (Invitrogen, USA). The purified protein was run on SDS-PAGE and Western blot with 3-OST-1 antibodies (Santa Cruz Biotechnology, Inc., USA).

3-OST-1 Activity Measurements

The sulfotransferase activity of 3-OST-1 produced in *B. subtilis* and *B. megaterium* was measured using an adaptation of a photometric coupled enzyme assay. This assay measures sulfo group transfer by aryl sulfotransferase IV (AST IV) from a sacrificial sulfo donor, *p*-nitrophenylsulfate (PNPS), to an acceptor molecule, 3'-phosphoadenosine 5'-phosphate, which is generated by 3-OST-1 transferring a sulfate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a polysaccharide substrate [10]. Briefly, cultures were grown, and overexpression of 3-OST-1 was induced as previously described; then, the cultures were sonicated in buffer containing Tris–HCl, NaCl, and 50 mM arginine and glutamic acid to increase solubility during concentration [11]. Cell lysate was then centrifuged, and the supernatant, which contained the soluble protein fraction, was concentrated 10-times using 10 kDa centrifugal filter (Millipore, USA) and incubated at 37 °C for 1 h with PAPS, PNPS, 50 mM MES buffer, heparan sulfate, AST IV, MgCl₂, and MnCl₂. Photometric absorbance measurements were taken at 400 nm every minute using a 96-well plate reader, and changes in absorbance were converted to micromolar PNP formed using the extinction coefficient $\epsilon=10.5\times 10^3$ [12].

Measurements of Endotoxin Concentration

Endotoxin concentration was assayed with the Limulus amoebocyte lysate (LAL) assay gel clot method (Associates of Cape Cod, Inc., USA). Briefly, the samples from *B. subtilis*,

B. megaterium, and *E. coli* were diluted and incubated with LAL and compared to stock solutions of endotoxin.

Results

Expression of 3-OST-1

The expression of 3-OST-1 was induced in *B. subtilis* and *B. megaterium*, and the protein was purified under denaturing conditions, and its identity was confirmed with Western blotting. Successful expression of 3-OST-1 expression in *B. subtilis* and *B. megaterium* is shown in Fig. 1a. When compared to non-induced cultures, the induced cultures express

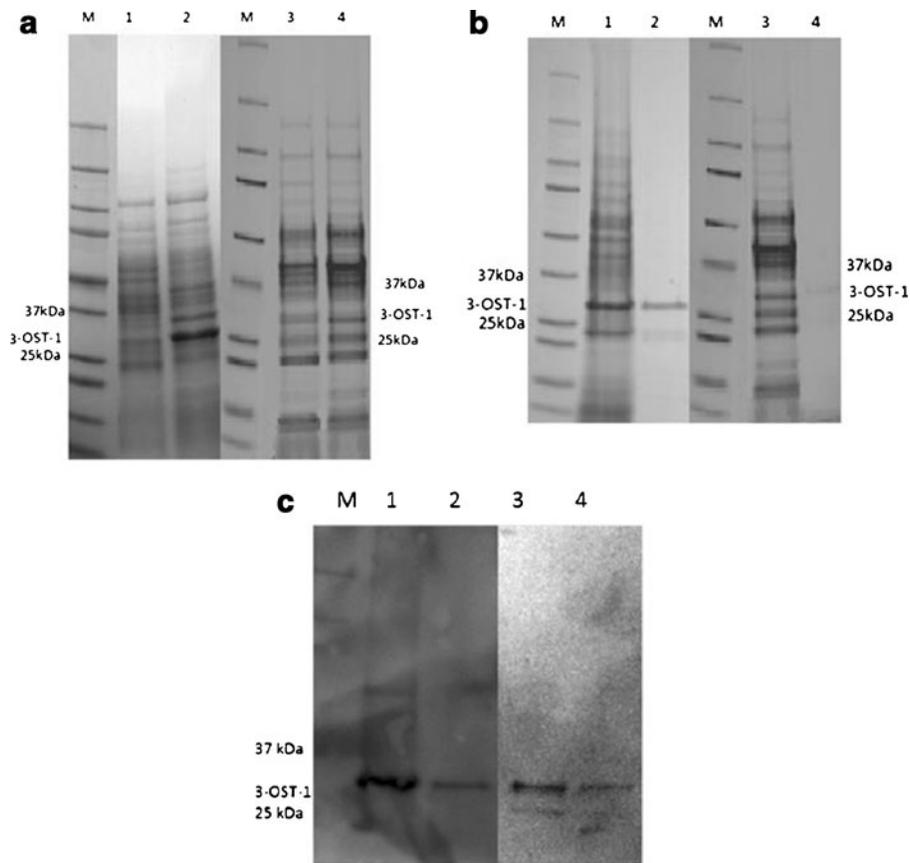


Fig. 1 The expression and purification of 3-OST-1 from *B. subtilis* and *B. megaterium* on SDS-PAGE and Western blotting. **a** SDS-PAGE of cell lysates. *M*, protein ladder; 1, *B. megaterium* pT7-3-OST-1 non-induced; 2, *B. megaterium* pT7-3-OST-1 induced; 3, *B. subtilis* without pHT01-3-OST-1; 4, *B. subtilis* pHT01-3-OST-1 induced. **b** SDS-PAGE. *M*, protein ladder; 1, *B. megaterium* pT7-3-OST-1 induced cell lysate; 2, *B. megaterium* pT7-3-OST-1 purified by elution under denaturing conditions; 3, *B. subtilis* induced pHT01-3-OST-1 cell lysate; 4, *B. subtilis* pHT01-3-OST-1 purified by elution under denaturing conditions. **c** Western blot. *M*, protein ladder; 1, induced *B. megaterium* pT7-3-OST-1 cell lysate; 2, *B. megaterium* purified 3-OST-1; 3, induced *B. subtilis* pHT01-3-OST-1 cell lysate; 4, *B. subtilis* purified 3-OST-1

significantly more 3-OST-1. Protein of the same size was also eluted from the nickel column during the 6× His-tag purification under denaturing conditions in both *B. megaterium* and *B. subtilis* (Fig. 1b), although the expression is quite low in *B. subtilis* as expected. Furthermore, these bands were also shown to be 3-OST-1 by Western blotting with 3-OST-1 antibodies (Fig. 1c).

Protein sequence analysis by LC-MS/MS conclusively determined that the overexpressed protein is 3-OST-1. The results from the protein analysis by LC-MS/MS of in-gel digested 3-OST-1 produced in *B. subtilis* are shown in Table 2. Peptides in the digested protein band that were identified by LC-MS/MS covered 54 % of the recombinant 3-OST-1 sequence, with a Masco score of 132, conclusively identifying the protein as recombinant murine 3-OST-1.

Activity of Expressed Enzyme

The 3-OST-1 produced in *B. megaterium* and *B. subtilis* was found to be enzymatically active. The coupled reaction assay showed that after induction, protein samples from *B. megaterium* (Fig. 2a) and *B. subtilis* (Fig. 2b) show a markedly higher sulfotransferase activity. Importantly, the sulfotransferase activities were found to be similar to those of the enzyme when it is produced in *E. coli* (Fig. 2c). To compare the sulfotransferase activity to that in *E. coli*-expressed 3-OST-1 constructed previously in another lab [8], we negated the background sulfotransferase activity by subtracting out the measurements from non-induced samples. The measurements for *B. megaterium* are not in units of micromolar PNP/milligram protein because no protein concentration measurement was taken as we were only looking for activity qualitatively. The specific enzymatic activity of the cell lysate of *B. subtilis* expressing 3-OST-1 was 0.3 U/mg, and that of *E. coli* lysate was found to be 0.5 U/mg, and the approximate specific activity of lysate of *B. megaterium* expressing

Table 2 Protein sequence analysis of 3-OST-1 expressed in *B. subtilis* by LC/MS

AA position	Detected peptide sequence	Observed <i>m/z</i>	Charges
105–124	DPSERVLSDYTQVLYNHLQK	1,202.63	2
210–229	GFYCLRDSGKDRCLHESKGR	1,163.04	2
194–209	LSPQINASNIFYFNKTK	935.52	2
238–251	LLDKLHEFYFHEPNK	593.65	3
163–176	FFPLGHHIIVDGDR	811.92	2
151–162	SLYHAHMLNWL	770.88	2
177–190	LIRDPFPEIQKVER	870.99	2
99–109	LLLILRDPSER	662.90	2
77–85	TPAYFTSPK	1,011.52	1
260–264	TFDWH	705.30	1
252–255	KFFK	569.34	1
86–89	VPER	500.28	1
256–259	LVGR	444.29	1
22–25	GGTR	390.21	1

Score, 132; coverage, 54 %

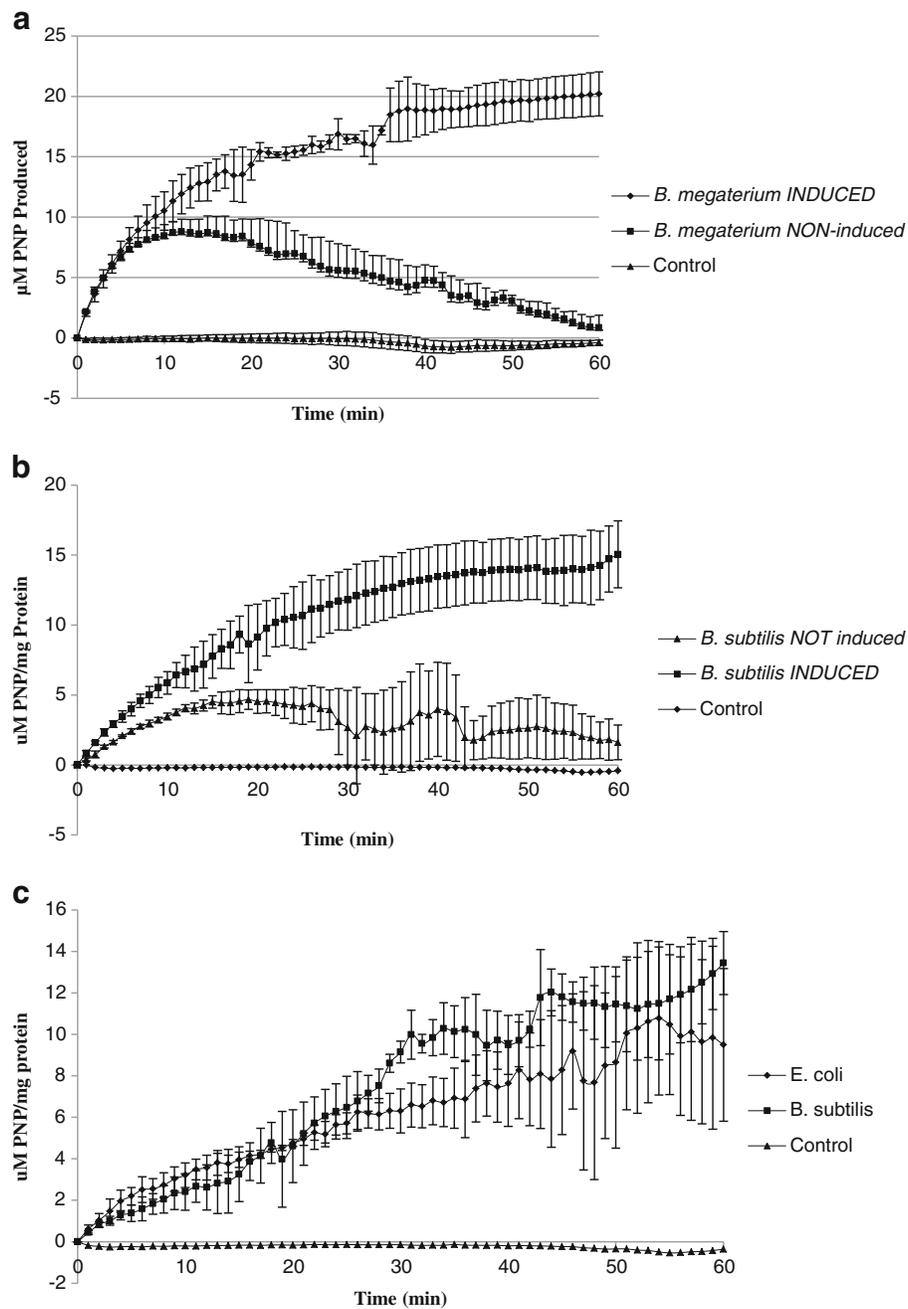


Fig. 2 Sulfotransferase activity of 3-OST-1 produced in *B. megaterium* and *B. subtilis*. Negative control is run with no protein. **a** Assay of sulfotransferase activity of 3-OST-1 produced in *B. megaterium*. **b** Assay of sulfotransferase activity of 3-OST-1 from *B. subtilis*. **c** Comparison of sulfotransferase activity of 3-OST-1 produced in *B. subtilis* and *E. coli*

3-OST-1 is similar to these values. These measurements further suggest that the protein we have induced is 3-OST-1 and, importantly, show that it is enzymatically active.

Endotoxin Levels of Expressed Enzyme

The LAL endotoxin concentration measurements are summarized in Table 3. These results indicate that 3-OST-1 collected from *E. coli* contains approximately 4×10^6 endotoxin units (EU) per mL. The *Bacillus*-expressed enzymes have drastically lower endotoxin unit per milliliter levels. *B. subtilis* was found to have 10^5 -fold less endotoxin, and *B. megaterium* was found to have at least 10^4 -fold less than *E. coli*.

Discussion

We have produced enzymatically active 3-OST-1, a critical enzyme for bioengineered synthesis of nonanimal source heparin in the Gram-positive bacteria *B. subtilis* and *B. megaterium*. While the enzyme had previously been expressed in *E. coli*, the endotoxin produced by the Gram-negative *E. coli* is not appropriate for the purpose of 3-OST-1 [4]. *B. subtilis* and *B. megaterium* were chosen to be ideal Gram positive and, thus, endotoxin-free hosts for the production of 3-OST-1.

While we were able to express 3-OST-1 in both *B. subtilis* and *B. megaterium*, the expression was quite low in *B. subtilis*. There are a number of possible reasons that contribute to this low expression. *B. megaterium* is an ideal host largely because it lacks alkaline proteases produced by *B. subtilis* that can degrade heterologous protein [7]. However, strains of *B. subtilis* that are deficient in numerous proteases have been developed, which make them a more improved host for heterologous protein expression [13]. Another explanation for the increased expression in *B. megaterium* is that we used a very strong promoter, the T7 promoter. Furthermore, we were unable to purify the protein with a nickel column under native conditions. We suspect that this is because the expressed protein is found largely in insoluble inclusion bodies, because we can purify it under denaturing conditions. Using a maltose-binding protein or another fusion tag that increases solubility may increase our yield of purified 3-OST-1 from *B. subtilis* and *B. megaterium*.

These *Bacillus* species have also been found to be excellent hosts for the expression of heterologous proteins targeted for secretion into the growth medium [14]. Secretion of 3-OST-1 and the other enzymes required for synthesis of bioengineered heparin could have

Table 3 The endotoxin concentration of 3-OST-1 expressed in *Bacillus* sp. and *E. coli*

Sample	Gel clot assay (EU/mL) ^a	Chromogenic assay (EU/mL) ^a
3-OST-1 from <i>E. coli</i>	$\sim 4 \times 10^6$	$> 4 \times 10^6$
Supernatant for <i>B. subtilis</i> cell lysate ^b	64±0	72.9±9.3
3-OST-1 from <i>B. subtilis</i>	64±0	48.4±2.4
3-OST-1 from <i>B. megaterium</i>	250	n.d.

^a All the values are the average of three replicates

^b Supernatant from *B. subtilis* cell lysate was used as a control, and it was prepared as follows: the pellet of *B. subtilis* was collected by centrifugation, resuspended in lysis buffer, and sonicated to obtain the cell lysate. Supernatant was obtained after centrifugation of the cell lysate

a tremendous impact on industrial production of the enzymes and bioengineered heparin. This should aid in protein recovery by avoiding protein aggregation that results in inclusion bodies.

Importantly, the levels of endotoxin found in the 3-OST-1 samples produced in these *Bacillus* species were found to be drastically lower than those when expressed in *E. coli*. Because of the pharmaceutical nature of the role of 3-OST-1, it is important that endotoxin concentration is minimized so that the resulting anticoagulant heparin meets USFDA standards. The levels we have measured are very low, and we believe that through optimization of reaction conditions, the levels will reach the required standards.

We have shown here that the enzymes required for the production of nonanimal source heparin can be expressed in Gram-positive, low-endotoxin *B. subtilis* and *B. megaterium*. The production of these enzymes with low endotoxin concentrations is an essential step towards the synthesis of bioengineered heparin.

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