Engineering *Bacillus megaterium* strains to secrete cellulases for synergistic cellulose degradation in a microbial community

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Title: Engineering *Bacillus megaterium* strains to secrete cellulases for synergistic cellulose degradation in a microbial community

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Abstract
Recent environmental concerns have intensified the need to develop systems to degrade waste biomass for use as an inexpensive carbon source for microbial chemical production. Current approaches to biomass utilization rely on pretreatment processes that include expensive enzymatic purification steps for the requisite cellulases. We aimed to engineer a synthetic microbial community to synergistically degrade cellulose by compartmentalizing the system with multiple specialized *Bacillus megaterium* strains. EGI1, an endoglucanase, and Cel9AT, a multimodular cellulase, were targeted for secretion from *B. megaterium*. A small library of signal peptides (SPs) with five amino acid linkers was selected to tag each cellulase for secretion from *B. megaterium*. Cellulase activity against amorphous cellulose was confirmed through a series of bioassays, and the most active SP constructs were identified as EGI1 with the LipA SP and Cel9AT with the YngK SP. Activity of the optimized cellulase secretion strains was characterized individually and in tandem to assess synergistic cellulytic activity. The combination of EGI1 and Cel9AT yielded higher activity than either single cellulase. Coculture of EGI1 and Cel9AT secreting *B. megaterium* strains demonstrated synergistic behavior with higher activity than either monoculture. This cellulose degradation module can be further integrated with bioproduct synthesis modules to build complex systems for the production of high value molecules.

Keywords: protein secretion, signal peptides, cellulose degradation, synthetic biology, microbial communities, *Bacillus megaterium*

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With a growing need for renewable energy sources, lignocellulosic biomass has emerged as a polysaccharide waste material with the potential to serve as a viable carbon source for cell systems [1, 2]. Cellulose, one of the primary components of lignocellulosic biomass, can be enzymatically broken down into its constitutive glucose
sugars. These sugars can serve as a feed source for bioengineered bacterial systems to synthesize a wide range of products, such as biofuels and pharmaceuticals [3, 4, 5]. However, current approaches to biomass utilization often rely on pretreatment processes and expensive enzyme purification schemes [2, 6, 7]. In some pretreatment schemes, the biomass is processed at high temperatures and extreme pH conditions in the presence of ionic liquids, to remove the lignin from the cellulosic biomass that will be targeted for degradation [8, 9]. The resulting amorphous cellulose can be degraded enzymatically with a combination of three cellulase activities – an endoglucanase, a celllobiohydrolase, and a beta-glucosidase, depicted in Figure 1 [10]. Efficient cellulolytic enzymes have been identified in the industrially relevant fungal species, that are expressed and purified into a cellulase cocktail for cellulose degradation, with the final sugars fed to a microbial production strain [2, 9, 11, 12]. In order to streamline the process of cellulose degradation and utilization, we aimed to build a microbial system for secretion of the requisite cellulases in a single-pot system where the production strain can thrive.

Many research groups have pursued methods for bioengineered cellulase production with the construction of cellulosomes, where large complexes of enzymes are secreted to the surface of the cell or to a scaffold, or with free enzyme cellulase secretion, using well-characterized and high efficiency enzymes which were naturally secreted or engineered for secretion [13, 14, 15, 16, 17]. In systems that naturally degrade biomass, cellulose is synergistically degraded by a community of organisms secreting cellulases with different activities [9, 18, 19]. This synergistic cellulolytic activity is crucial for efficient cellulose degradation, and can be retained in both the cellulosome strategy as well as with free enzyme secretion [6, 14, 20]. While the cellulosome activity may benefit from the proximity of the different enzymatic sites, this effect can be achieved with free enzyme secretion of multimodular enzymes [14, 21, 22]. In the free enzyme secretion, without a scaffold restricting the enzyme movement, the cellulases may function simultaneously at different sites of the cellulose microfibrils, and thereby synergistically degrade the material [10]. With the goal of constructing a bacterial system with efficient cellulase secretion, we focused on incorporating free enzyme secretion with smaller cellulases instead of secreting a large scaffolded unit.

To engineer a microbial community for cellulose degradation, a host organism for cellulase expression and secretion was required. With the aim to integrate a microbial production strain in a single system with the cellulose degradation module, we chose to target a bacterial strain for cellulase secretion. While protein secretion has been engineered in Gram-negative species, more success has been seen with Gram-positive organisms where proteins can more easily cross the single cellular membrane while retaining protein folding and activity [23, 24, 25]. Naturally secreted proteins are tagged with a signal peptide sequence at the N-terminus that triggers secretion by the Sec or Tat pathway; these signal peptides can be used to tag recombinant proteins for secretion [24, 26, 27].

*Bacillus megaterium*, as a large Gram-positive bacterium with a natural signal peptide secretion machinery and low extracellular protease activity, was selected to be engineered for cellulase secretion [28, 29]. *B. megaterium* has been used to express many recombinant proteins [22, 23, 30]. Some of these, including a levansucrase and an endoglucanase, were tagged with signal peptides and secreted at levels of 1-2 mg/L [28, 30, 31]. For the secretion of recombinant proteins in Gram-positive organisms, tagging the proteins of interest with signal peptides has been effective, but requires screening...
large libraries with up to 400 independent signal peptide tags to identify constructs for successful secretion of a protein while also retaining protein activity [32, 33, 34, 35, 36, 37]. In this study, a signal peptide library was engineered with linkers between the signal peptides and the enzymes of interest, to improve the likelihood of identifying successful secretion constructs without the need to screen a large library. To increase the yield of extracellular protein, the MS941 strain was engineered to remove the major extracellular protease of *B. megaterium*, a neutral metallo-protease (NprM), resulting in as low as 1.4% of the natural extracellular protease activity [38]. In this study, the *B. megaterium* MS941 strain was engineered to secrete each cellulase for the cellulose degradation module.

As recombinant microbial technologies have allowed for large pathways of enzymes or groups of synergistic enzymes to be cloned into individual bacterial hosts, an adverse metabolic effect has been observed where metabolic burden can overwhelm the host and lead to inefficient enzyme expression [39, 40, 41, 42, 43]. To address this challenge, microbial communities can be engineered to synergistically perform complex tasks, by dividing the demands among multiple specialized organisms, to maximize the efficiency of desired product formation and to allow the metabolic load to be distributed throughout the system [18, 40, 44, 45, 46, 47, 48]. Modeling the cellulose degradation system after the microbial consortia that naturally degrade cellulosic biomass, we expressed each cellulase with signal peptide secretion tags in independent *B. megaterium* strains. The activity of the secreted cellulases was characterized under different conditions and in tandem to identify synergistic behavior, which was further explored in cocultures of the *B. megaterium* strains. We aimed to combine signal peptide enzyme secretion and microbial communities to engineer a bacterial system with cellulolytic capabilities.
Figure 1. Schematic depicting cellulose degradation via three enzymatic steps: (1) endoglucanase degradation of amorphous cellulose, (2) cellobiohydrolase degradation of crystalline cellulose (cellodextrin), and (3) beta-glucosidase cleavage of cellobiose, for the final product of glucose. EGI1 is an endoglucanase that can target amorphous cellulose for degradation, while Cel9AT is a multimodular cellulase that can fully degrade cellulose into glucose monomers [22, 30]. The endoglucanase step is the expected rate-limiting step of the cellulose degradation pathway. Therefore, including EGI1 and Cel9AT together may result in synergistic degradation of cellulose.

Results and Discussion

Engineered cellulase signal peptide libraries for protein secretion in *B. megaterium*. To engineer a bacterial system for efficient cellulose degradation, *B. megaterium* was selected as the bacterial host for expression and secretion of the cellulase enzymes. Two cellulases previously expressed in *Bacillus* were chosen – the endoglucanase EGI1 and the multimodular cellulase Cel9AT [22, 30]. EGI1 was previously expressed in *Bacillus subtilis* with a native *Bacillus akiibai* signal peptide, showing secretion of about 2 mg/L [30]. For this project, the *egi1* gene was commercially generated by Genscript. Cel9AT is a truncated form of the multimodular cellulase Cel9ACel48A, which was previously expressed in *B. megaterium* and demonstrated degradation of cellulose into glucose [22]. The activity of these enzymes is depicted in Figure 1, which shows that EGI1 can complete the first step of the degradation pathway, while Cel9AT can degrade cellulose into glucose monomers. EGI1 can target the rate limiting step of the cellulose degradation pathway to work synergistically with Cel9AT to degrade cellulose.

We engineered secretion of these two cellulases in *B. megaterium* using five signal peptide (SP) tags known to trigger natural protein secretion from *B. megaterium* [49]. The 5 SPs, denoted as α-amy, LipA, NprM, YocH, and YngK, were targeted due to high levels of expression and secretion of the natural proteins. Each of these SPs were prepared with unique linker regions of five amino acids following the SP. The linker sequences corresponded to the first five amino acids of the naturally secreted *B. megaterium* proteins, which was previously determined to be the most effective linker length [49]. The EGI1 cellulase was cloned into each signal peptide construct, with the SP and linker positioned at the N-terminus and the 6x histidine tag at the C-terminus. A construct with no SP tag was cloned to act as a non-secreting control. The same cloning approach was used to construct a Cel9AT SP library with the LipA, NprM, YocH, and YngK signal peptides. The final SP libraries were transformed into *B. megaterium* strains to be screened for cellulase secretion and activity.
Figure 2. Schematic depicting protein, linker, and signal peptide. For secretion through the Sec pathway, the ribonuclease signal recognition particle (SRP) targets proteins for secretion during translation. The SRP-protein complex binds to a translocase complex on the cell membrane. Signal peptidases cleave the signal peptide after translocation across the membrane for protein release into the extracellular space [21, 50, 51].

Expression of cellulase signal peptide libraries identified constructs for secretion of active EGI1 and Cel9AT in LB media. To assess the secretion and cellulase activity of the signal peptide library, each construct was expressed in rich media and the cellulase activity in the supernatant was characterized. The EGI1 signal peptide library was expressed in LB media, and all supernatants were concentrated 50x and buffer exchanged to remove any residual sugars. Relative EGI1 secretion was assessed using SDS PAGE to visualize the protein bands, presented in Figure S1 of the supporting materials. EGI1 was identified by size at 89 kDa, and ImageJ analysis was used to quantify the EGI1 protein using protein concentrations of the ladder as a reference (Figure 3a) [52]. For each of the EGI1 signal peptide constructs, EGI1 expression was detected. For all SP constructs except the EGI1 LipA, higher EGI1 secretion was detected when protein expression was induced with xylose. In the case of EGI1 LipA, the uninduced sample indicated higher secretion than the induced condition (Figure 3a). Highest EGI1 secretion was detected with uninduced EGI1 LipA at 108 (± 2) mg/L. The highest EGI1 secretion from an induced SP construct was in the YocH SP construct, with cellulase secretion at 106 (± 2) mg/L. In the supernatant of the noSP EGI1 strain, no significant enzyme secretion was detected (< 4 mg/L in the concentrated supernatant), indicating that successful secretion occurred only in the SP tagged constructs. Activity of the library was assessed using the DNS assay to detect degradation of the carboxymethyl cellulose substrate (CMC) [53, 54, 55, 56]. As shown in Figure 3c, highest cellulose degradation was seen with the induced EGI1 YocH at 4.8% (± 0.3) substrate conversion. In the uninduced EGI1 LipA, 4.0% (± 0.4) substrate conversion was detected. Expression in the LB media condition demonstrated similar levels of secretion and activity in the uninduced EGI1 LipA and the
induced EGI1 YocH, where highest secretion correlated with highest activity. For all of the expression cases, higher activity was detected in the signal peptide constructs compared to the noSP control with a CMC degradation at 0.7% (±0.04). The most active SP constructs showed 6-fold and 7-fold higher cellulolytic activity.

The same techniques were implemented to characterize the Cel9AT SP library expressed in LB media. Figure S1 illustrates an SDS PAGE image with the Cel9AT protein bands highlighted at 62 kDa. Protein concentrations were quantified using ImageJ analysis, and are shown in Figure 3b. The noSP negative control strain indicated no detectable secretion of Cel9AT. Cellulase expression was detected in the Cel9AT LipA and Cel9AT YngK strains. The highest Cel9AT secretion was identified at 27 (±1) mg/L with the induced Cel9AT YngK SP construct. As seen with the EGI1 library, the LipA construct demonstrated high expression in the uninduced case, with 22 (±6) mg/L of Cel9AT secreted. No detectable extracellular protein was observed in the noSP control. Cellulase activity was quantified using the DNS assay, and cellulose degradation is presented in Figure 3d. Highest cellulose degradation was identified in the uninduced Cel9AT LipA construct with 3.6% (±0.2) CMC degradation. The induced Cel9AT YngK showed 3.0% (±0.4) cellulose degradation, indicating that the samples with highest protein secretion also showed the highest levels of cellulolytic activity. Comparing expression of the two libraries indicated significantly higher expression of the EGI1 library than the Cel9AT library in LB media. Both EGI1 and Cel9AT were secreted at highest levels with the uninduced LipA construct, while the YocH SP was only effective for EGI1 secretion and not Cel9AT, reflecting the importance of screening both uninduced and induced expression conditions with several different SPs.
Expression of cellulase signal peptide libraries identified constructs for secretion of active EGI1 and Cel9AT in M9+ minimal media. To further characterize the secretion and cellulase activity of the signal peptide library constructs, the EGI1 SP library was expressed in M9+, a minimal media optimized for *B. megaterium* growth. A minimal media condition may be advantageous for our system as it allows the carbon source to be carefully defined, and cell growth in this media can be optimized to reach higher cell densities than the LB rich media allows. All constructs were expressed, and EGI1 was quantified in concentrated supernatant samples by SDS-PAGE analysis, with
the SDS PAGE shown in Figure S2 and the EGI1 protein concentrations shown in Figure 4a. EGI1 expression in M9+ was highest from the induced EGI1 LipA construct at 6 (± 5) mg/L, which was 18-fold lower than the highest secretion detected previously in LB media at 108 (± 2) mg/L. In contrast with the LB expression cultures, the uninduced EGI1 LipA did not show detectable secretion. Secretion was also detected in the induced EGI1 YocH construct at 3 (± 2) mg/L. Figure 4c shows the activity of each protein sample, with highest cellulolytic activity detected in the induced EGI1 LipA construct at 2.2% (± 0.2) CMC degradation. The induced EGI1 YocH exhibited lower CMC degradation at 1.6% (± 0.3), which correlated to the decrease in secreted protein concentration. While the cellulase concentration in the induced EGI1 α-amy construct was below the detection limit of the SDS PAGE analysis, the DNS assay showed cellulolytic activity of the induced EGI1 α-amy at 2.0% (± 0.1) CMC degradation. This discrepancy may be a result of the higher sensitivity of the DNS assay than the SDS PAGE and ImageJ technique, with the latter unable to accurately detect low concentrations of protein. In all signal peptides, higher secretion and activity was detected in the induced cases, and there was negligible activity in the noSP construct.

Figure 4b shows the quantified protein concentrations for the Cel9AT SP library expression in M9+ media (with the relevant SDS PAGE shown in Figure S2). While the EGI1 library demonstrated higher cellulase secretion in the LB media, the Cel9AT constructs showed significantly higher secretion in M9+ media than in LB media. Highest Cel9AT secretion was detected in the induced Cel9AT YngK construct at 52 (± 7) mg/L. The uninduced Cel9AT LipA strain secreted 43 (± 6) mg/L of Cel9AT, reflecting secretion with this construct in both media conditions as well. With the noSP construct, low levels of Cel9AT were detected at 4 (± 1) mg/L, and could be the result of cell lysis at the end of culture growth. Figure 4d presents the observed CMC degradation, with highest activity detected in the induced Cel9AT YngK sample at 4.5% (± 0.3). Similar levels of activity were detected in the uninduced and induced cultures of the LipA, NprM, and YngK, between 3.9% and 4.5% CMC degradation. The activity detected in the noSP control was lower, at around 3.0% (± 0.5), but significantly higher than the activity detected in the noSP construct expression in the EGI1 library and the LB media conditions. Of the two enzymes, Cel9AT demonstrated higher cellulolytic activity at low concentrations, and the minimal media condition may have been more favorable to maintain stability and activity of the Cel9AT during expression, resulting in a high activity despite a low concentration of enzyme. The strains capable of highest secretion and activity in both media conditions were the EGI1 LipA construct and the Cel9AT YngK construct, which were subsequently selected and screened for synergistic behavior.
Figure 4. Cellulase signal peptide library expression in M9+ minimal media. (a) EGI1 protein concentrations detected in concentrated supernatants of EGI1 SP library, (b) Cel9AT protein concentrations detected in concentrated supernatants of Cel9AT SP library, (c) Cellulose degradation from concentrated supernatants of EGI1 SP library, (d) Cellulose degradation from concentrated supernatants of Cel9AT SP library. Lanes are alternating with uninduced (green) and induced (white) samples. Each pair is a different signal peptide condition, with the first two samples presenting the no SP negative control. All supernatants were concentrated 50x and buffer exchanged to remove any residual sugars from the media. Each sample was expressed in biological duplicates, and activity was screened with technical replicates in triplicate. All bars represent mean values, with error bars denoting ± standard deviation.

EGI1 and Cel9AT demonstrated different preferred conditions for cellulolytic activity that were balanced for synergistic behavior. The EGI1 LipA and Cel9AT YngK strains were selected to express EGI1 and Cel9AT to identify reaction conditions for cellulolytic activity of each enzyme. To produce the enzymes, EGI1 LipA was cultured and expressed in LB media while Cel9AT was expressed in M9+ media. Each cellulase was expressed separately, concentrated, buffer exchanged and quantified using SDS PAGE and ImageJ analysis. Mixed enzyme reactions were prepared, testing a range of enzyme concentrations between 0 mg/L and 2.5 mg/L, emulating concentrations of the
enzymes that were previously secreted into the supernatant (prior to concentrating). The measured cellulolytic activity of each reaction is presented in Figure 5. At pH 5.0 (Figure 5a), with Cel9AT at 0 mg/L, even the highest EGI1 concentration of 2.5 mg/L showed no detectable activity (<0.04%). In contrast, without EGI1, the 2.5 mg/L Cel9AT condition demonstrated 1.4% (± 0.5) CMC degradation, indicating that for the single enzyme reactions, Cel9AT had a higher detectable activity. With 2.5 mg/L of Cel9AT, adding increasing amounts of EGI1 increased cellulose degradation from 1.4% (± 0.5) to 2.5% (± 0.02). With 2.5 mg/L of EGI1, adding increasing amounts of Cel9AT increased cellulose degradation from below the detection limit (<0.04%) to 2.5% (± 0.02), indicating a greater impact from addition of Cel9AT than EGI1. This trend was observed at each concentration of EGI1 and Cel9AT in Figure 5a. Figure 5b presents cellulase activity at pH 6.0, where single enzyme reactions indicated a reversal in the behavior of the two enzymes. When Cel9AT was at 0 mg/L, the highest concentration of EGI1 led to cellulose degradation that was detectable at 0.2% (± 0.02), in contrast with pH 5.0 where the activity was below detection. Without EGI1, the 2.5 mg/L concentration of Cel9AT led to a cellulose degradation of 0.1% (± 0.1), demonstrating significantly lower activity than at pH 5.0. The difference in enzyme behavior at pH 6.0 was visible in the mixed enzyme reactions as well. When the Cel9AT concentration was held constant at 2.5 mg/L, increasing the EGI1 concentration had a significant effect, particularly when the EGI1 concentration increased from 0.5 mg/L up to 1.25 mg/L, where the cellulose degradation increased from 0.9% (± 0.08) up to 3.8% (± 0.1). For comparison, when EGI1 was held constant at 2.5 mg/L, increasing the Cel9AT concentration from 0.5 mg/L up to 1.25 mg/L only resulted in a degradation increase from 2.3% (± 0.2) to 3.1% (± 0.1). This trend was consistent throughout the data set, and indicated that at the pH 6.0 condition, addition of EGI1 had a higher impact on cellulolytic activity than addition of Cel9AT. Results from both pH conditions demonstrated that each enzyme had preferred reaction conditions, and that combinations of low concentrations of both enzymes resulted in significant increases in activity. While Cel9AT had a higher impact on the activity at lower pH, and EGI1 had a higher impact on activity at higher pH, both enzymes were active at both reaction conditions.
Figure 5. Cellulose degradation from reactions with different concentrations of EGI1 and Cel9AT. All reactions were at a specified pH condition, (a) pH 5.0 (green) or (b) pH 6.0 (blue). Cellulose degradation was measured using the DNS assay. Activity was screened with technical replicates in triplicate. All bars represent mean values, with error bars denoting ± standard deviation.

Synergistic cellulolytic activity detected in reactions with different ratios of EGI1:Cel9AT. To characterize the synergistic behavior of EGI1 and Cel9AT over a range of conditions, cellulolytic activity of different ratios of EGI1 to Cel9AT was measured with total cellulase concentration held constant at 5 mg/L. The cellulase concentrations correlate to those that can be secreted from Bacillus megaterium strains. Higher levels of CMC degradation were observed in reactions at 37 °C as compared to 30 °C (Figure 6). Each pH condition further impacted the activity of the two enzymes. At pH 5.0, highest activity was observed when more Cel9AT was present than EGI1, specifically in the EGI1:Cel9AT ratios of 4:6 and 3:7, resulting in cellulose degradation between 2.4% and 2.5%. At pH 6.0, highest activity was detected when both enzymes were present over a wide range of different ratios between 9:1 and 4:6, resulting in CMC degradation between 1.4% and 1.7%. In the final condition, the pH 7.0 reactions demonstrated highest cellulolytic behavior with higher quantities of EGI1 than Cel9AT. The highest activity was observed in the 8:2 ratio with 2.2% (± 0.5) cellulose degradation. In all of the different pH and temperature conditions tested, each set showed highest activity when both EGI1 and Cel9AT were present rather than purely EGI1 or Cel9AT, with up to a 6-fold increase in the cellulolytic activity indicating a synergistic cellulose degradation effect.
Figure 6. Cellulose degradation from reactions with different ratios of EGI1: Cel9AT, with 5 mg/L total cellulase concentration. (a) Reactions at 30 °C (green), (b) reactions at 37 °C (blue). Cellulose degradation was measured using the DNS assay. Activity was screened with technical replicates in triplicate. All bars represent mean values, with error bars denoting ± standard deviation.

Synergistic cellulase activity achieved in Bacillus coculture of EGI1 and Cel9AT strains. Bacillus megaterium strains were expressed to determine whether the synergistic cellulose degradation observed between EGI1 and Cel9AT could be retained in coculture. Three inoculation ratios were tested for the EGI1 LipA and Cel9AT YngK constructs to compare the protein expression in an EGI1 monoculture, a 1:1 coculture of EGI1: Cel9AT, and a Cel9AT monoculture, expressed in both LB rich media and M9+ minimal media. We note that the Cel9AT monoculture condition demonstrated secretion of this multimodular cellulase, which has not been reported previously. In addition, the EGI1 monoculture expression yielded 3.4 mg/L of EGI1 secretion in the supernatant prior to concentrating, which is 70% higher than previous secretion efforts using Bacillus subtilis [30]. The protein concentrations of EGI1 and Cel9AT in the concentrated supernatants of each culture are presented in Figure 7a. Higher cellulase secretion was
detected in the LB than the M9+ expression cultures, with the highest LB cellulase expression yielding 171 (± 6) mg/L in the EGI1 monoculture, compared to the highest M9+ cellulase expression yielding 25 (± 6) mg/L in the Cel9AT monoculture under the given expression conditions. In the LB coculture system inoculated with both the EGI1 and Cel9AT strains, EGI1 was secreted at 78 (± 6) mg/L and Cel9AT at 25 (± 2) mg/L, for a total cellulase production of 103 (± 8) mg/L. Figure 7b indicates higher cellulase activity observed in the LB cultures than the M9+ cultures, as expected from the higher cellulase secretion in LB. The EGI1 monoculture in LB demonstrated 4.4% (± 0.1) cellulose degradation, while the coculture of EGI1 and Cel9AT showed highest activity at 5.1% (± 0.7) cellulose degradation. The highest cellulolytic activity was found in the coculture condition, despite higher cellulase secretion detected in the EGI1 monoculture, confirming the retention of synergistic cellulolytic activity of EGI1 and Cel9AT in coculture.

**Figure 7.** Monoculture and coculture expression with different inoculation ratios of EGI1:Cel9AT and different media conditions. (a) Cellulase concentrations of EGI1 (blue) and Cel9AT (white) in concentrated monoculture and coculture supernatants, (b) Cellulose degradation from concentrated monoculture and coculture supernatants. All supernatants were concentrated 50x and buffer exchanged to remove any residual sugars from the media. Each sample was expressed in biological duplicates, and activity was screened with technical replicates in triplicate. All bars represent mean values, with error bars denoting ± standard deviation.

**Discussion and conclusions.** EGI1 and Cel9AT were expressed and secreted by *B. megaterium* by screening libraries of signal peptide constructs for each cellulase. The success of the small SP libraries is attributed to the short amino acid linkers incorporated between the SP and the enzyme for each construct. By following the SPs with linkers corresponding to the first five amino acids of the naturally secreted proteins, the resulting cleavage sites match the naturally secreted proteins and may contribute to the secretion with each tested SP. This success suggests that including these linkers between the SPs and the target enzyme could allow for secretion of other proteins using *B. megaterium* as
a host. Comparing the success of the EGI1 and Cel9AT libraries also indicates that the specific combination of SP and enzyme affects the amount of protein that accumulates in the supernatant. While EGI1 secretion was highest with the LipA SP tag, the Cel9AT secretion was highest with the YngK secretion tag. Screening a small SP library with amino acid linkers for each SP allows for a higher likelihood of successful secretion at the desired level. Comparison of the expression of the different signal peptide constructs demonstrates that certain SP and enzyme combinations may present tightly regulated, inducible expression, such as with the EGI1 α-amy expression in LB media, while some constructs will show expression independent of induction, as seen with the Cel9AT YngK expression in M9+ media. This expression behavior has been seen previously with these \textit{B. megaterium} expression plasmids, and is characteristic of the T7 promoter system. Additionally, the EGI1 constructs showed higher secretion and activity in the LB media condition, while Cel9AT expression was highest in the M9+ media, indicating that different enzyme and SP combinations demonstrated different activity depending on the media condition. These behaviors allowed for the SP to serve as a controlled variable, where the degree and control of expression of a target enzyme can be modified by using alternate SP constructs. Specific SP constructs could be incorporated in cocultures depending on the desired expression conditions. The LB media condition would allow for a coculture system including the high EGI1 expression strains, while selecting the minimal media with a controlled carbon source could better integrate the Cel9AT producing strains. Identifying the constraints of the system would guide selection of the specific SP constructs to incorporate. The SP linker libraries present a new approach to engineer secretion of recombinant proteins in \textit{B. megaterium} to be integrated into synthetic microbial communities.

Activity of the secreted EGI1 and Cel9AT enzymes was assessed under many reaction conditions including several temperatures, pH, and ratios of the two enzymes, to establish synergistic cellulose degradation. Testing at different pH conditions revealed that while Cel9AT shows higher activity at low pH (pH 5.0) and EGI1 shows higher activity at high pH (pH 7.0), all pH conditions showed higher activity from a combination of both enzymes when compared to activity from a single cellulase. These results demonstrate cellulosolytic behavior of each enzyme varies depending on the conditions of the final system. The synergistic behavior exhibited by combinations of EGI1 and Cel9AT presents a cellulose cocktail for cellulose degradation, similar to the cellulase cocktails integrated for industrial biomass utilization. Characterizing both the enzyme expression and the enzyme activity of each strain facilitated assembly of a coculture system with synergistic cellulosolytic capabilities. In the LB coculture with the 1:1 inoculation ratio, synergistic activity of EGI1 and Cel9AT was achieved with higher activity than detected in the monocultures. These results indicate the improved cellulose degradation through cocultures of the two \textit{B. megaterium} strains. Expression of the monocultures and cocultures revealed that total cellulase protein secreted varies between the different medias, inoculation ratios, and culture conditions. Due to the variability of cellulase expression profiles, identifying the culture conditions with highest activity will require investigating a wider range of inoculation ratios and expression conditions. With the requisite cellulosolytic enzymes secreted, the \textit{B. megaterium} supernatant can be harvested and concentrated to act as a cellulose degradation slurry, without the need for expensive enzyme purification. Establishing expression conditions for higher cellulase titers is crucial for producing sufficient enzyme in the coculture supernatant to degrade
cellulose and support growth of a bioproduction strain. In this scheme, the cellulase coculture would grow with a bioproduction strain in a synthetic community capable of efficient cellulose utilization. The coculture system conditions would need to be screened to balance growth and activity of all the microbial strains, to function with highest efficacy. In the cellulose degradation coculture, balancing the secretion of the two proteins as well as the activity of the two enzymes requires careful tuning of the system conditions. Introducing a product synthesis strain will place further demands on the system, in order to identify conditions that retain the cellulase production and activity while supporting the needs of the bioproduction strain. Building a community with many different strains provides the advantage of many tunable organisms and components, to allow system regulation by manipulating controllable variables. Our findings illustrate the value of introducing *B. megaterium* as an engineerable organism for synthetic microbial communities, to build tunable systems with new functionalities.

Methods

**Bacterial strains, media, and growth conditions.** All bacterial strains used in this study are listed in Table S1 in the supporting materials. *E. coli* and *B. megaterium* were grown on Lysogeny broth (LB) agar, in LB broth (Becton Dickinson (BD)), or in M9+ with glucose (1x M9 salts (48 mM Na₂HPO₄, 22 mM KH₂PO₄, 18.8 mM NH₄Cl, 8.6 mM NaCl), 0.1 mM CaCl₂, 2 mM MgSO₄, 36 µM FeSO₄, 4.14 µM MnSO₄, 1% glucose). All strains were cultured at 37 °C with shaking at 225 rpm. Antibiotics used were ampicillin (Amp) at a concentration of 100 µg/ml, tetracycline (Tet) at 40 µg/ml, and chloramphenicol (Cam) at 10 µg/ml.

**Signal peptide library plasmid construction and transformation.** All plasmids, bacterial strains, and primers are listed in Table S3 in the supporting materials. The commercial pPT7 and pT7-RNAP shuttle vectors were used for recombinant gene expression in *B. megaterium* (MoBiTec GmbH). The signal peptide library constructs were provided by Dr. Nick Marchand in the Collins Lab at RPI, with the following five constructs: pPT7-SPα-amy, pPT7-SPLipA, pPT7-SPNprM, pPT7-SPYocH, and pPT7-SPYngK [49]. The empty plasmid pPT7 was used for the noSP construct cloning. All signal peptide sequences are provided in Table S4 in the supporting materials. EGI1 was PCR amplified from pUC57-EGI1 (a commercially synthesized gene from Genscript) using the primers EGI1_Fwd_Primer and EGI1_Rev_His_Primer [49]. These genes were cloned between the SpeI and SalI restriction sites of the five signal peptide library constructs: pPT7-SPα-amy, pPT7-SPLipA, pPT7-SPNprM, pPT7-SPYocH, and pPT7-SPYngK. The no signal peptide construct was cloned using the primers EGI1_noSP_Fwd_Primer and EGI1_Rev_His_Primer, with the NheI and SalI restriction sites. All pPT7 vectors contain antibiotic resistance genes for Amp (*E. coli*) and Tet (*B. megaterium*). Cel9AT was PCR amplified from pCHis1622-CbCel9ACel48A, provided by Dr. Isaac Cann’s group at UIUC, using the primers Cel9A_Fwd_Primer and Cel9A_Rev_His_Primer [30]. These genes were cloned between the SpeI and SalI restriction sites of the five signal peptide library constructs: pPT7-SPα-amy, pPT7-SPLipA, pPT7-SPNprM, pPT7-SPYocH, and pPT7-SPYngK. The noSP construct was cloned using the primers Cel9A_noSP_Fwd_Primer and Cel9A_Rev_His_Primer, with the NheI and SalI restriction sites. These vectors contain antibiotic resistance genes for Amp (*E. coli*) and Tet (*B. megaterium*). All of the vectors were cloned and characterized in DH5α *E. coli* cells using standard molecular biology techniques. All of the engineered
derivatives of the pPT7 plasmid were transformed into MS941 cells alongside the pT7-
RNAP plasmid to allow for xylose-inducible T7 expression of the target gene constructs.

**Signal peptide library growth and protein expression.** All protein expression was
in the *Bacillus megaterium* host. For each signal peptide library construct, colonies of
*Bacillus megaterium* were picked and used to inoculate 5 mL of LB media in 15 mL
curved bottom culture tubes. These were left overnight shaking at 37 °C, 225 rpm, at an
angle of ~55°, for a maximum of 12 hrs. These conditions minimize the cells that settle at
the bottom of the tube, and allow for consistency with expression. Expression cultures of
50 mL were grown in 250 mL Erlenmeyer flasks, and 500 mL were grown in 2.5 L
baffled Erlenmeyer flasks. The 50 mL cultures were inoculated with 1 mL of overnight
culture, while the 500 mL cultures were inoculated with 10 mL of overnight culture.
These were grown for ~4-6 hrs, and induced at an absorbance measured at 600 nm to be
0.250. Cultures were induced with 1 mL or 10 mL of 25% xylose, respective to original
culture volume. After induction, protein expression occurred overnight (~15-20 hrs)
shaking at 37 °C, 225 rpm. Cultures were centrifuged at 4800 rcf, 4 °C, for 1 hr or more,
to separate the pellet from the supernatant. Supernatant was sterile filtered to separate out
solid particulates. With the 50 mL cultures, the supernatant was spun down to concentrate
50-fold using 30 kDa ultra-15 (50 mL tube) ultrafiltration membranes. In the larger
culture volumes, tangential flow filtration using a 30 kDa Viva Flow 200 membrane was
applied to concentrated the sample. Filtrate was discarded while retentate held the protein
of interest. Protein was further buffer exchanged into M9+ media with no carbon source.
The resulting concentrated samples were detected via SDS PAGE, using 10% Novex
precast SDS gels and 1x Invitrogen MOPS buffer, for 60 min at 180 V. The resulting gels
were stained using the Invitrogen SimplyBlue SafeStain, and washed in distilled water.
To quantify the amount of the target proteins in the supernatant, the relative intensity of
the SDS PAGE bands was correlated to known protein concentrations. Concentrations of
bovine serum albumin (BSA) ranging from 50 mg/L to 2000 mg/L were assessed using
the SDS PAGE protocol, to serve as known standards for quantification with the Fiji
ImageJ software [52]. The resulting calibration was applied to quantify the protein bands
present in the Invitrogen SeeBlue Plus2 Prestained protein standard, which was used as a
reference to quantify all secreted cellulase concentrations. Final samples were diluted as
necessary into the M9+ buffer with no carbon source for reactions to measure cellulase
activity.

**Cellulase activity detection using the DNS assay.** Cellulase activity was detected
using the 3,5-dinitrosalicylic acid (DNS) assay, which detects reducing sugars [54, 55,
56]. The assay was optimized to function for a linear detection range at concentrations
equal to or greater than 100 µg/mL of glucose. For each reaction, a ratio of 1:1 substrate
(carboxymethyl cellulose, CMC) to cellulase was kept shaking at 225 rpm, at 37 °C for
48 hrs, unless otherwise specified. The standard reaction volume was 200 µL in a 2 mL
tube, which was taped to rest horizontally in the shaker to improve mixing and reaction
conditions. After the reaction duration was completed, the 200 µL reaction was combined
with 600 µL of DNS reagent to stop any remaining cellulase activity in the reaction. The
samples were capped and boiled in a heat block at 105 °C, for 10 min. The tubes were
then cooled on ice for 5 min or longer. From each sample, 100 µL was placed into a 96-
well, flat-bottom plate, with absorbance measured at 540 nm. A standard curve was used
to convert the absorbance measurement into a percent degradation of the CMC substrate.
**Associated Content**

Supporting Information
Detailed sequence information of signal peptide vectors. This material is available free of charge via the Internet at [http://pubs.acs.org](http://pubs.acs.org). Plasmids are available on Addgene.

Include the following:
- S1. SDS PAGE results of cellulase signal peptide library expression in LB rich media.
- S2. SDS PAGE results of cellulase signal peptide library expression in M9+ minimal media.
- S3. Table S3: Bacterial strains, plasmids, and primers for cloning cellulases into signal peptide constructs.
- S4. Table S4: Nucleotide and polypeptide sequences of signal peptides and linkers.

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K.Z.K., M.A.G.K., and C.H.C. conceived the study and designed the experiments; N.M. prepared the initial SP and linker plasmids; K.Z.K. performed the experiments with assistance from E.J.M. and K.M.R.; K.Z.K., M.A.G.K., and C.H.C. prepared the manuscript; and all authors contributed to the discussion of the research and interpretation of data.

Notes:
The authors declare no competing financial interests.

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**References**


