Drawing up a collaborative contract: Amino acid cross-feeding between inter-species bacterial pairs

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ABSTRACT

Synthetic microbial communities have the potential to enable new platforms for bioproduction of biofuels and biopharmaceuticals. However, using engineered communities is often assumed to be difficult because of anticipated challenges in establishing and controlling community composition. Cross-feeding between microbial auxotrophs has the potential to facilitate co-culture growth and stability through a mutualistic ecological interaction. We assessed cross-feeding between 13 *Escherichia coli* amino acid auxotrophs paired with a leucine auxotroph of *Bacillus megaterium.* We developed a minimal media capable of supporting the growth of both bacteria and used the media to study co-culture growth of the 13 interspecies pairs of auxotrophs in batch and continuous culture, and on semi-solid media. In batch culture, eight of thirteen pairs of auxotrophs were observed to grow in co-culture. We developed a new metric to quantify the impact of cross-feeding on co-culture growth. Six pairs also showed long-term stability in continuous culture, where co-culture growth at different dilution rates highlighted differences in cross-feeding amongst the pairs. Finally, we found that cross-feeding-dependent growth on semi-solid media is highly stringent and enables identification of the most efficient pairs. These results demonstrate that cross-feeding is a viable approach for controlling community composition within diverse synthetic communities.

KEYWORDS: Microbial community, cross-feeding, co-culture, synthetic ecology

INTRODUCTION

When using mono-clonal populations of microbes for applications in biotechnology, synthetic biologists and metabolic engineers often struggle to increase yield and productivity without overwhelming cellular metabolism or drastically reducing cell fitness (Shong, Jimenez Diaz, & Collins, 2012). Further, mono-clonal systems are often limited by the functional capabilities, both inherent and engineerable, of specific species. In synthetic microbial communities, functions from multiple strains or species can be combined and the system can be designed to distribute metabolic burden between individual community members (Tsoi et al., 2018). Many examples of co-cultures that outperform mono-clonal systems have been recently reported (Bernstein et al., 2012; Goyal et al., 2011; Guo et al., 2020; Tsai et al., 2010; Xia et al., 2012; H. Zhang & Stephanopoulos, 2016; H. Zhang & Wang, 2016). For example, (Jones et al., 2016) found that dividing the production of flavan-3-ols between two *E. coli* strains resulted in a 970-fold increase in production.

While microbial communities present new opportunities for bioproduction (Cavaliere et al., 2017), significant challenges remain to enable their broad adoption for biochemical engineering applications, including coordinating the behavior of the organisms and enabling reliable and predictable community growth and composition (Kong et al., 2018; H. Zhang & Wang, 2016). Cell-cell communication systems, primarily based on microbial quorum sensing, have been engineered to enable coordinated behaviors of different strains and species (Kylilis et al., 2018; Marchand & Collins, 2013; Moon, Lou, Tamsir, Stanton, & Voigt, 2012; Ping et al., 2015). In bio-sensing applications, cell-cell communication has enabled the distribution of sensing and actuating functions between strains (Dinh et al., 2020; Ping et al., 2015; Ravichandar et al., 2017; Stephens et al., 2019) and enabled the integration of multiple inputs via biological computation (P. Du et al., 2020; Moon et al., 2012; Tamsir et al., 2011). To date, manipulating community composition in communities used for bioproduction has primarily relied on tuning inoculation ratios and growth conditions (Goyal et al., 2011; Jones et al., 2016;

Kalbarczyk et al., 2018; H. Zhang & Wang, 2016). Synthetic biologists have explored a range of strategies to control community dynamics and composition, such as quorum-sensing induced cell death (Balagaddé et al., 2008) or antibiotic resistance (Hu et al., 2010). Mutualistic interactions, where cooperation yields co-culture growth, hold significant potential for bioproduction systems requiring the growth of multiple organisms because these strategies do not require the addition of antibiotics. Further, by not relying on QS to control growth, the functions of the organisms in co-culture can be coordinated via communication.

Environmental conditions, such as media composition (Klitgord & Segrè, 2010; Ma et al., 2018; J. Zhang et al., 2011) and spatial organization (Said & Or, 2017), have been observed to yield cooperative growth in laboratory-grown microbial co-cultures. This is often because the co-existing species are auxotrophs and must obtain one or more essential nutrient from the environment. Auxotrophy in bacteria has been observed to facilitate spontaneous cross-feeding relationships (D'Souza et al., 2014; Hosoda et al., 2011; Mee et al., 2014; Pande et al., 2015; Park et al., 2011), which is a type of mutualistic interaction where auxotrophic organisms exchange metabolites from other members in their community. Despite the potential of cross-feeding to enable cooperative growth of multispecies engineered systems, efforts to date have most often focused on pairing auxotrophic strains of the same species such as *E. coli* (Mee et al., 2014; Park et al., 2011; Wintermute & Silver, 2010) and yeast (Shou, Ram, & Vilar, 2007). Previous work has shown that cross-feeding between amino acid auxotrophs of *E. coli* and a second Gram-negative organism, *Zymomonas mobilis,* yielded co-culture growth through sharing of essential amino acids (Kosina et al., 2016).

In this work, we investigate spontaneous obligate cross-feeding between two industrially relevant bacterial hosts, Gram-negative *E. coli* and Gram-positive *Bacillus megaterium. E. coli* is ubiquitous in biochemical engineering applications. *B. megaterium* has been used for small molecule production including vitamin B_{12} (Korneli et al., 2013) and penicillin acylase (Yang et al., 2006), has a

highly efficient protein secretion system (Stammen et al., 2010), and low extracellular protease activity (Kuhn & Fortnagel, 1993; K. D. Wittchen & Meinhardt, 1995). Here, we tested 13 auxotrophic pairs using a leucine *B. megaterium* auxotroph and 13 *E. coli* amino acid auxotrophs from the Keio collection (Baba et al., 2006). In order to characterize this behavior, we first developed a minimal media to support the growth of both species. We then investigated spontaneous mutualistic cross-feeding behavior in batch culture with various inoculation concentrations. To better understand the resulting data, we developed a cross-feeding efficiency metric to describe cross-feeding performance. Additionally, we assessed community stability of our pairs through continuous culture at different dilution rates. Finally, we demonstrated semi-solid agar plate assays as a screening tool for identifying strong cross-feeding pairs.

MATERIALS AND METHODS

Bacterial strains and starter cultures

Bacterial strains are listed in Table 1. Bacterial cultures were grown overnight in 5 ml LB at 37°C and 225 RPM, where *E. coli* cultures were supplemented with 50 µg/mL kanamycin and *B. megaterium* cultures were grown without antibiotics. All overnight cultures were washed twice with 1X phosphate buffered saline (PBS) before inoculation of growth assay media. Media development and cross-feeding experiments, including mono-culture growth assays, were performed in the absence of antibiotics.

Media development

Washed cells were inoculated into 3 ml B3 media ($1.4x10^{-2}$ M K_2HPO_4 , $1.8x10^{-2}$M KH_2PO_4, $6.2x10^{-3}$ M $(NH_4)_2HPO_4$, $1.7x10^{-3}$ M $MgSO_4$, $3.6x10^{-5}$ M $FeSO_4$, and $4.1x10^{-5}$ M $MnSO_4$) or M9 media ($4.8x10^{-2}$ M Na_2HPO_4, $2.2x10^{-2}$M KH_2PO_4, $1.9x10^{-1}$ M NH_4Cl, $8.6x10^{-3}$ M NaCl, $2.0x10^{-3}$ M $MgSO_4$, and $1.0x10^{-4}$ M $CaCl_2$) supplemented with 0.5% glucose and 0.5% sucrose. M9 media were supplemented with $MnSO_4$ and $FeSO_4$ as indicated. OD600 was measured every 4 to 6 hours with an Envision multilabel plate reader (Perkin- Elmer).

Batch growth assays

Washed cells were inoculated into 3 ml M9+ (M9 with 4.1 µM $MnSO_4$ and 36 µM $FeSO_4$) supplemented with 0.5% glucose and 0.5% sucrose in deep 48-well plates as mono- and co-cultures at varying inoculation concentrations of *B. megaterium* and *E. coli* strains. 48-well plates were set at a 20° angle to limit *B. megaterium* settling at 225 RPM. Samples were collected every 24 hours for four days and were enumerated through serial dilution in 1X PBS with 0.1% leucine and spot plating. Samples were plated on MacConkey agar for selective growth of *E. coli* and M9+ with sucrose for selective growth of *B. megaterium*.

Continuous culture assays

We built a multiplexed continuous culture platform based on the system described by Miller *et al.* with minor modifications (Miller et al., 2017). Septum-stoppers (VWR) were used in place of silicone stoppers on reaction vessels, silicone stoppers were used on sterile media carboys, and polyetheretherketone (PEEK) aeration lines were used in the place of stainless steel needles inside the reactor. Reaction vessels were filled with M9+ media supplemented with 0.125% glucose and 0.125% sucrose and heated to 37 °C on a dry heat block prior to inoculation. A 500-fold dilution from the overnight cultures was achieved by inoculating with 1 mL washed cells in PBS via injection with a 3 ml sterile syringe and 18G sterile needle. Multichannel peristaltic pumps (Ismatec) were initially set at a dilution rate of 0.035 hr^{-1} for 84 hours to allow cell growth in an isochoric phase. The pumps were then set to a dilution rate of 0.15 hr^{-1} or 0.075 hr^{-1}. Samples were taken every 24 hours and were enumerated via serial dilution and spot plating on selective agar as described above.

Semi-solid media growth assays

Cells from overnight cultures were washed twice and diluted 500-fold in 1X PBS. For co-cultures, the diluted cells were mixed at a 50:50 ratio. Five µL of the cells were pipetted for each mono-culture and

co-culture condition onto semi-solid M9+ media supplemented with 0.5% glucose, 0.5% sucrose, and 1.5% agar. Digital photographs of the plates were captured following incubation at 37 °C for 72 hours

RESULTS

Media development

Before studying spontaneous obligate cross-feeding between amino acid auxotrophs of *B. megaterium* and *E. coli,* we first developed a minimal media that supports high-level growth of both organisms. M9 medium, a popular and commercially available minimal medium, does not support *B. megaterium* growth (Fig. 1B), while *E. coli* reaches an OD_{600}>0.6 by 24 h (Fig. 1C). B3 medium (Fig. 1A, (Lankford et al., 1966; Marchand & Collins, 2013)), supported growth of *B. megaterium* reaching OD_{600}>0.8 (Fig. 1B), while *E. coli* grown in B3 is reduced 60% compared to M9 (Fig. 1C). Both media have phosphate buffers and ammonium as a nitrogen source; however, the concentration of ammonium in B3 is 16-fold less than that in M9. The main differences in the composition of these media are that M9 contains Ca^{2+}, while B3 contains Fe^{2+} and Mn^{2+}. The importance of Fe^{2+} and Mn^{2+} was assessed by growing *B. megaterium* in B3 media without $FeSO_4$ and $MnSO_4$ (B3-). *B. megaterium* growth above background was not observed in B3- media (Fig. 1B), leading us to hypothesize that *B. megaterium* growth in M9 media could be improved by supplementation with $FeSO_4$ and $MnSO_4$.

To assess growth of *B. megaterium* and *E. coli* in M9 supplemented with $FeSO_4$ and $MnSO_4$, each species was inoculated in mono-culture into 3 mL M9 supplemented with 4.1 µM $MnSO_4$ and 3.6, 36 or 360 µM $FeSO_4$. $MnSO_4$ concentration was also titrated initially; however, poor growth was observed with 41 and 410 µM $MnSO_4$, likely due to toxicity (data not shown). As shown in Figure 1B, *B. megaterium* growth in M9 media supplemented with $MnSO_4$ and either concentration of $FeSO_4$ was indistinguishable from *B. megaterium* growth in B3 media. *E. coli* growth in M9 supplemented with $FeSO_4$ and $MnSO_4$ was similar to what was observed in M9 without supplementation (Fig. 1C). Here, a relatively simple approach was successfully used to identify a set of new minimal media that support the growth of both

E. coli and *B. megaterium* at levels comparable to minimal media specialized for each organism. M9 supplemented with 4.1 µM $MnSO_4$ and 36 µM $FeSO_4$ was renamed M9+.

Mutualistic cross-feeding-dependent co-culture growth in batch culture

To assess cross-feeding between *E. coli* and *B. megaterium*, mono- and interspecies co-culture growth of 13 *E. coli* amino acid auxotrophs from the Keio collection (Baba et al., 2006) and a *B. megaterium* leucine auxotroph (MS942) was quantified in batch culture. All strains were grown overnight in LB media and washed twice with 1X PBS before inoculation. Three mL of M9+ supplemented with 0.5% glucose and 0.5% sucrose was inoculated at approximately 10^4 CFU/ml *B. megaterium* and 10^6 CFU/ml *E. coli.* Cells were cultured in 48-deep well plates at 37 °C for 4 days. Samples were obtained every 24 hours and viable cells were quantified by serial dilution and spot plating on selective media. Figure 2 shows the growth results for the thirteen interspecies pairs in co-culture as well as mono-culture growth for all strains.

The number of viable cells in the monoculture of the *B. megaterium* leucine auxotroph decreased every 24 hours after inoculation and fell below the detection limit of 10^3 CFU/ml by 96 hours (Fig 2, blue, MC, all panels). In contrast, we observed stable concentrations of viable cells for 9 of 13 *E. coli* auxotrophs in mono-culture (Fig. 2, orange, MC). A decrease in viable cell density was observed for mono-cultures of the *E. coli* tryptophan, histidine and cysteine auxotrophs in mono-culture. However, the density decreased by no more than 50-fold over 4 days. The phenylalanine auxotroph did not exhibit the expected behavior in mono-culture, where it was observed to grow on its own to concentrations up to 10^9 CFU/mL.

We observed an increase in cell density for 8 of 13 interspecies pairs in co-culture. The amount of co-culture growth varied significantly amongst the *E. coli* auxotrophs (Fig 2, orange, CC) when co-cultured with the B. megaterium leucine auxotroph (Fig 2, blue, CC). We observed final cell densities similar to those for the prototrophic parent strains in M9+ media (10^9 CFU/mL *E. coli* and 10^8 CFU/mL *B.*

megaterium) for the *E. coli* proline and isoleucine auxotrophs in co-culture. Both organisms were also observed to increase in cell density for the co-cultures with the *E. coli* phenylalanine, methionine, arginine, tyrosine and lysine auxotrophs. For the co-cultures with the *E. coli* tryptophan and glutamine auxotrophs, subtle increases in biomass compared to mono-culture conditions were observed. Finally, the co-cultures with the *E. coli* histidine, threonine, serine, and cysteine auxotrophs did not show any improvements over mono-culture growth. For serine and cysteine, the *E. coli* cell densities were lower in co-culture than in mono-culture, possibly due to competition with the *B. megaterium* cells. We also assessed the growth of co-cultures of the prototrophic *B. megaterium* strain MS941 with each of the *E. coli* auxotrophs. We observed that all 13 of the *E. coli* auxotrophs grew in the presence of *B. megaterium* MS941 (Supplementary Figure 1).

To quantify cross-feeding dependent growth of the auxotrophic pairs, we developed an expression for Cross-feeding Efficiency (CFE) that compares the changes in cell densities for each organism in co-culture and mono-culture (Equation 1). Specifically, the maximum change in the $\log_{10}$ of cell density observed at 3 or 4 days for the monoculture is subtracted from the maximum change in the $\log_{10}$ of cell density for the same strain in co-culture. This calculation is repeated for the partner strain and the two terms are summed. Using this equation, we quantitatively ranked the cross-feeding success of each of our tested pairs. The tabulated results are show in Fig. 3A. The first column of Figure 3A shows CFE values calculated from the growth data using the initial *B. megaterium*:*E. coli* inoculation ratio of 10^4:10^6 CFU/ml. These results capture the trends of our growth data with the proline, methionine, and isoleucine *E. coli* auxotrophs paired with the leucine auxotrophic *B. megaterium* having the highest CFE values. The CFE calculations enable quantitative comparisons of cross-feeding dependent growth between different auxotrophic pairs and between auxotrophic pairs grown with different inoculation ratios. To examine the effect of inoculation concentration on cross-feeding, batch experiments were repeated at four additional inoculation ratios of *B. megaterium* to *E. coli* 10^4:10^4,

10^4:10^5, 10^5:10^5, 10^5:10^6 CFU/mL (see Supplementary Figures 2 to 5 for growth data). CFE was calculated for all 13 auxotrophic pairs at each inoculation ratio (Fig. 3A). Cross-feeding between auxotroph pairs was most successful at the higher inoculation ratios, most notably those with *E. coli* starting at ≥10^5 CFU/ml. As shown in Figure 3A, overall the cross-feeding efficiency was lower for all auxotrophic pairs at 10^4:10^4 CFU/ml relative to all other inoculation ratio, where 3.7±1.0 was the highest CFE observed. This suggests that there is a minimum inoculation concentration required to establish cross-feeding dependent growth of these pairs. With the exception of the tyrosine auxotroph of *E. coli,* increasing the starting concentration of *B. megaterium* from 10^4 to 10^5 led to an increase in the CFE for every auxotrophic pair.

Equation 1

$$\boldsymbol{CFE} = (\Delta_{max}\boldsymbol{E.coli\ CC} - \Delta_{max}\boldsymbol{E.coli\ MC}) + (\Delta_{max}\boldsymbol{B.megaterium\ CC} - \Delta_{max}\boldsymbol{B.megaterium\ MC})$$

To compare co-culture performance across the different inoculation concentrations, the CFE was normalized by the maximum CFE observed at a given inoculum (CFE_n, Fig. 3B). Based on growth performance in the 10^4:10^6 pairs, we determined that $CFE_n \geq 0.4$ is indicative of at least some cross-feeding-dependent mutualistic growth. The cross-feeding pairs that consistently achieved $CFE_n \geq 0.4$ across all inoculation ratios, with the exception of 10^4:10^4, were *E. coli* auxotrophic for proline, isoleucine, methionine, phenylalanine, arginine, and tyrosine paired with leucine auxotrophic *B. megaterium*. The CFE_n of the *E. coli* methionine and phenylalanine auxotrophs in co-culture increased as a function of inoculum concentration. Among the other co-cultures, $CFE_n \geq 0.4$ was achieved for lysine and tryptophan auxotrophs of *E. coli* in co-culture for inoculation ratios of 10^5:10^5 and 10^5:10^6 CFU/ml, co-cultures with *E. coli* auxotrophic for cysteine at an inoculation ratio of 10^5:10^5 CFU/ml, and the *E. coli* auxotrophic for serine co-cultured with *B. megaterium* at inoculation ratio of 10^5:10^6 CFU/ml. When the inoculation ratio of 10^4:10^6 CFU/ml was used the pairs that grew to prototrophic cell densities or that

grew in co-culture while the corresponding *E. coli* mono-cultures experience cell death were found to have $CFE_n \geq 0.8$, representing a strong cross-feeding partnership. *E. coli* auxotrophic for proline and isoleucine in co-culture had $CFE_n \geq 0.8$ and grew to prototrophic cell densities in all inoculations except 10^4:10^4 CFU/ml. Methionine and arginine auxotrophs of *E. coli* paired with leucine auxotrophic *B. megaterium* had $CFE_n \geq 0.8$ and grew to prototrophic cell densities for 10^5:10^5 and 10^5:10^6 inoculations. $CFE_n < 0.4$ indicates little or no cross-feeding between partners. At an inoculation ratio of 10^4:10^4 CFU/ml, the low CFE_n values indicate poor cross-feeding in all tested auxotrophic pairs as compared to cross-feeding performance at higher inoculation ratios. At 10^4:10^4 CFU/ml, we observed CFEn < 0 for *E. coli* auxotrophic for histidine, serine, tryptophan and threonine which may indicate competitive interactions between partner strains.

Cross-feeding-dependent co-culture growth in continuous culture

In order to assess the use of cross-feeding to promote stable co-culture growth, we examined all 13 auxotrophic pairs using a 20-reactor continuous culture platform. Starter cultures were grown in LB and washed with 1X PBS prior to inoculation into M9+ supplemented with 0.125% glucose and 0.125% sucrose. The $FeSO_4$ concentration was decreased 10-fold to 3.6 μM to reduce precipitation in the carboys and tubing. Following inoculation, the reactors were operated under isochoric conditions for approximately 84 hours at a dilution rate of $0.035 hr^{-1}$. The dilution rate was then increased to 0.15 hr^{-1} and the reactors were operated under continuous culture conditions for five days. Cell concentrations were determined by spot plating on selective media.

Figure 4 shows the results of the continuous culture experiments for one representative biological replicate of each auxotrophic pair and the auxotrophic strains in mono-culture. At least two biological replicates were tested for all mono-culture and co-culture conditions. At least four biological replicates were characterized for all cultures where viable cells were observed after two days of continuous culture. The number of viable cells in the leucine *B. megaterium* auxotroph monoculture

(Fig. 4, dashed blue line, all graphs) remained approximately constant throughout the isochoric phase and decreased to levels below the detection limit of 10^3 CFU/ml within 24 hours of starting continuous culture, likely due to washout. The monocultures of the *E. coli* histidine, isoleucine, lysine, serine, and tyrosine auxotrophs (Fig. 4, red dashed lines) behaved similarly. The monocultures of the *E. coli* methionine, phenylalanine, proline, and tryptophan auxotrophs all showed a decrease in viable cell density during the isochoric phase and washed out within 24 hours of continuous culture. Small increases in cell density were observe for the *E. coli* arginine, cysteine, glutamine, and threonine auxotrophs, but all monocultures washed out to below the detection limit within 48 hours of continuous culture.

While co-culture growth was observed for eight pairs of auxotrophs in batch culture, sustained growth in continuous culture was observed for only the isoleucine, proline, and methionine pairs. For these three pairs, the *E. coli* auxotroph grew to a cell density of approximately 10^8 CFU/ml and the leucine *B. megaterium* auxotroph reached approximately 10^7 CFU/ml within 48 hours of continuous culture, and these cell densities were maintained for the remainder of the experiment. The co-culture with the phenylalanine auxotroph, which showed increased co-culture growth in batch culture as well as some mono-culture growth, was observed to wash out below detection within 48 hours of continuous culture. The co-cultures of the tyrosine, tryptophan, serine, threonine, and cysteine *E. coli* auxotrophs with the leucine *B. megaterium* auxotroph also washed out within 48 hours of continuous culture. We observed intermediate behavior for the co-cultures of the lysine, glutamine, arginine, and histidine auxotrophs. For these four pairs, the co-cultures were observed to survive for at least three days of continuous culture. However, lower cell densities of about 10^5 CFU/ml E. coli and 10^3-10^4 CFU/ml B. megaterium were achieved in continuous culture and wash out occurred between day three and five. In one of four biological replicates of the co-cultures with lysine and histidine auxotrophs, sustained co-culture growth was observed (data not shown).

To further characterize the four intermediate pairs, we repeated the continuous culture assays at a halved dilution rate of 0.075 hr^{-1}. Two biological replicates were chracterized and the growth data for one replicate is shown in Figure 5. As expected, the viable cells in all of the *B. megaterium* and *E. coli* mono-cultures were observed to decrease below the detection limit during continuous culture. Sustained co-culture growth was observed for each of the pairs at the reduced dilution rate, with both organisms achieving similar cell densities between 10^6 and 10^7 CFU/mL. While some variation in cell density was observed, co-culture growth was observed in all cases for the duration of the experiment.

Semi-solid media assay for cross-feeding

Finally, we examined cross-feeding between the auxotrophic pairs on semi-solid media. *E. coli* and *B. megaterium* auxotrophs were grown in LB overnight, washed twice with PBS, and pipetted onto M9+ supplemented with 0.5% sucrose, 0.5% glucose and 1.5% agar. To examine co-culture growth, cells were mixed prior to plating and growth was compared visually to the mono-cultures after 3 days at 37 °C (Fig. 6).

As expected, growth was not observed for the *B. megaterium* and *E. coli* auxotrophs in monoculture, with the exception of the phenylalanine auxotroph. Consistent with the batch culture results, however, more biomass was observed when the phenylalanine auxotroph was cultured with the *B. megaterium* leucine auxotroph than in mono-culture. Co-culture biomass production was observed for five of the remaining 12 *E. coli* auxotrophs: arginine, isoleucine, methionine, proline and tyrosine. The six pairs that showed enhanced biomass in co-culture on the semi-solid media also had the highest CFE values in batch culture at all inoculation ratios except 10^4:10^4. The auxotrophic pairs with the most biomass on the plates, methionine, isoleucine and proline, were able to grow at the highest dilution rate under continuous culture. However, strains observed to have lower CFE values that are able to grow in continuous culture at lower dilution rates, including histidine, glutamine and lysine were not observed

to grow in the assay. These results suggest that growth assays on semi-solid defined media can be used to efficiently screen auxotrophic pairs for their ability to cross-feed and grow in co-culture.

DISCUSSION

In this study we developed a defined minimal medium capable of supporting the growth of both Gram-negative *E. coli* and Gram-positive *B. megaterium.* Using this medium, M9+, we characterized cross-feeding dependent growth between auxotrophic inter-species pairs of bacteria in batch and continuous culture, as well as on semi-solid agar. M9+ has broad use as a minimal media for *B. megaterium* growth for bioproduction and protein expression (Kalbarczyk et al., 2018; Williams et al., 2019), co-culture growth of *B. megaterium* with *E. coli* (Marchand & Collins, 2013) and as selective agar for quantification of *B. cereus* (Bhagwat et al., 2020).

We showed that spontaneous mutualisms are formed and sustained between many pairs of amino acid auxotrophs of two industrially relevant hosts. When cultured with the prototrophic *B. megaterium* under batch culture conditions, all 13 *E. coli* auxotrophs were observed to grow in unsupplemented M9+. However, when grown with the *B. megaterium* leucine auxotroph, the *E. coli* auxotrophs of proline, isoleucine and methionine showed the most consistent and highest amount of across all culture modalities. Several *E. coli* auxotrophs showed intermediate growth, while four auxotrophs did not show any cross-feeding depending growth, when co-cultured with the *B. megaterium* leucine auxotroph. In our system, we found that there was a threshold inoculation concentration of 10^5 CFU/ml required for at least one organism for cross-feeding dependent growth to occur. This suggests that sufficient cells are required to establish the mutualistic interactions, where each strain provides the essential nutrient to their partner, or the cells will die despite the pair's ability to cross-feed. In the continuous culture experiments, the isochoric phase allows the cells to begin growing collaboratively before the pump is turned on and a higher growth rate is required to prevent washout. The continuous culture experiments demonstrated that these spontaneous partnerships are

stable over longer periods of time without any efforts to co-evolve the strains or engineer a cooperative behavior. This is especially noteworthy because the strains are not expected to co-occur in nature. Additionally, we showed that lower dilution rates could facilitate low-level cell density maintenance in poor cross-feeding pairs. These pairs with lower CFE may be good targets for future studies where laboratory evolution or engineering experiments could be used to increase co-culture growth. Cross-feeding-dependent growth on semi-solid agar plates was observed to be very stringent, likely due to lower mass-transfer rates compared to liquid culture. Our results indicate that the semi-solid assay could be used to screen large numbers of auxotrophic pairs for cross-feeding dependent growth.

The proline, isoleucine, and methionine *E. coli* amino acid auxotrophs showed the highest levels of cross-feeding dependent growth with the *B. megaterium* leucine auxotroph. Previous studies describing spontaneous cross-feeding in intra-species pairs of *E. coli* auxotrophs (Mee et al., 2014; Wintermute & Silver, 2010). Of particular note, Mee *et al.* co-cultured pairs of the same *E. coli* amino acid auxotrophs used in this study. High levels of co-culture growth were observed for more than half of the 13 co-cultures tested for each of the methionine, phenylalanine or lysine *E. coli* auxotrophs. They also found that pairings with proline auxotrophs rarely yielded cooperative co-culture growth, where co-culture growth was only observed with the highly cooperative phenylalanine and lysine auxotrophs. The differences between the identities of the auxotrophs observed to form the best intra-species and inter-species pairings suggests that the two species have differing preferences for cross-fed metabolites. This variability in preference between different species is not unexpected. Indeed, it is thought to facilitate diversity in natural microbial communities (Germerodt et al., 2016; Hoek & Merks, 2017). Future work to assess general trends in *B. megaterium* cross-feeding preferences should involve additional *B. megaterium* auxotrophs and inter- and intra-species co-culture with *E. coli* auxotrophs.

As we move towards building more complicated communities, we can use cross-feeding not only to facilitate mutualism for stable composition, but also take advantage of molecules secreted

naturally by different bacterial strains. Flux balance analysis was used to determine what molecules are costlessly secreted by 24 different species of bacteria, and their models predict costlessly secreted molecules in a range of different carbon sources and in the presence and absence of oxygen (Pacheco et al., 2019). The implication of natural costless secretion suggests new strategies for cross-feeding in communities for biotechnology as cross-fed metabolites that push flux towards your product of interest could be selected. Auxotrophy can also be used to fine tune community composition (Losoi et al., 2019; Ziesack et al., 2019), by engineering levels of metabolite secretion for auxotrophic pairs that fail to cross-feed spontaneously we can maintain that organism at a lower cell density within the community. We can also selectively supplement media to increase or decrease the cell density of an auxotroph, allowing for control of community composition. Lastly, we can use amino acid cross-feeding to explore cooperative ecological interactions in nature. For instance, our work suggests that auxotrophs of different species may preferentially cross-feed different amino acids, likely as a result of differential sharing and uptake of shared metabolites. However, to draw conclusions about natural cooperation and provide insights into the mechanisms and regulation of these processes, a larger library of inter-species cross-feeding interactions should be characterized in detail.

Synthetic ecological approaches can be used to overcome the challenges associated with growing microbes together in laboratory and industrial settings. Our work demonstrates not only that cross-feeding between strains that have been engineered only to be auxotrophic for an amino acid can be used to enable inter-species cooperative growth, but that the cross-feeding partnership remains stable with approximately constant ratios of cell density during continuous culture. This will enable engineers to harness the potential of synthetic communities for bioproduction through combining diverse bacteria, chosen or optimized for a specific function, where community composition is stabilized by easily enabled cross-feeding interactions.

ACKNOWLEDGEMENTS

We would like to acknowledge F. Meinhardt for providing *B. megaterium* MS942. This work was supported by the National Science Foundation (MCB-1055676 and CBET-1403815).

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Table 1. Bacterial strains used in this study, their auxotrophies, and gene knockouts.

Organism	Strain	Auxotrophy	Gene Knockout	Citation
B. megaterium	MS941	None	None	(K. D. Wittchen & Meinhardt, 1995)
	MS942	Leucine	Δ*leuB**	(Wittchen et al., 1998)
E. coli	BW25113	None	None	(Baba et al., 2006)
	JW2786-1	Arginine	Δ*argA*	(Baba et al., 2006)
	JW3582-2	Cysteine	Δ*cysE*	(Baba et al., 2006)
	JW3841-1	Glutamine	Δ*gluA*	(Baba et al., 2006)
	JW2004-1	Histidine	Δ*hisB*	(Baba et al., 2006)
	JW3745-2	Isoleucine	Δ*ilvA*	(Baba et al., 2006)
	JW2806-1	Lysine	Δ*lysA*	(Baba et al., 2006)
	JW3973-1	Methionine	Δ*metA*	(Baba et al., 2006)
	JW2580-1	Phenylalanine	Δ*pheA*	(Baba et al., 2006)
	JW0233-2	Proline	Δ*proA*	(Baba et al., 2006)
	JW2880-1	Serine	Δ*serA*	(Baba et al., 2006)
	JW0003-2	Threonine	Δ*thrC*	(Baba et al., 2006)
	JW1254-2	Tryptophan	Δ*trpC*	(Baba et al., 2006)
	JW2581-1	Tyrosine	Δ*tyrA*	(Baba et al., 2006)

*MS942 was reported to be a *leuC* knockout; however, Sanger sequencing revealed that *leuB* is inactivated.

Figure text:

Figure 1. Development of M9+ media for co-culture growth of *E. coli* and *B. megaterium*. (A) Molar concentrations of M9 salts and B3 salts. Growth of *B. megaterium* (B) and *E. coli* (C) on B3 (filled circles), B3- (empty circles, *B. megaterium* only), M9 (triangles), M9 medium with 4.1 µM $MnSO_4$ and 3.6 µM $FeSO_4$ (blue diamond), with 4.1 µM $MnSO_4$ and 36 µM $FeSO_4$ (green diamond) or 4.1 µM $MnSO_4$ and 360 µM $FeSO_4$ (red diamonds). Error bars represent standard deviation of three biological replicates.

Figure 2. Cell densities of mono-cultured (MC) and co-cultured (CC) auxotrophs of *B. megaterium* and *E. coli* in batch culture. *E. coli* were inoculated at approximately 10^6 CFU/ml and B. megaterium at approximately 10^4 CFU/ml and all cultures were grown for 96 hours. Cell densities were quantified every 24 hours, and the results are shown in $\log_{10}$CFU/ml. For each panel, the gene deleted in the *E. coli* strain is labeled, and cell densities are shown in shades of orange (E. coli) or blue (*B. megaterium* MS942 Δ*leuB,* all panels). Panels are organized from highest to lowest cross-feeding efficiency (CFE) value. Error bars represent one standard deviation from the mean for 3 biological replicates.

Figure 3. (A) Cross-feeding efficiency (CFE) of batch culture cross-feeding pairs for five inoculation concentrations (*B. megaterium*:*E. coli)*. Rows are labeled with the amino acid synthesis gene deleted from the *E. coli* strain. Columns show CFE values determined for co-cultures with *B. megaerium* MS942 Δ*leuB* at different inoculation concentrations. The standard deviation was calculated from three biological replicates per pairing. (B) All values here were calculated from the log of the colony forming units measured from selective plating. (B) Heat map of CFEn values for each inoculation ratio. CFEn

values were calculated by normalizing the CFE values by the maximum CFE observed for each inoculation ratio.

Figure 4. Comparison of monoculture and co-culture growth of auxotrophs of *B. megaterium* and *E. coli* in batch culture in continuous culture. Following inoculation, the reactors were operated isochorically for 3.5 days before 5 days of continuous culture at a dilution rate of 0.15hr^{-1}. The *E. coli* gene knockout is listed in the top right corner of panel. Cell densities were quantified every 24 hours and the $\log_{10}$CFU/ml is shown. *E. coli* cell densities are shown in red and *B. megaterium* cell densities are shown in blue. Monocultures are represented by dotted lines and cocultures are represented by solid lines. Plots are organized in the same order as in Figure 2. Each experiment was run in duplicate. One representative biological replicate is shown.

Figure 5. Monoculture and co-culture of intermediate cross-feeding pairs *B. megaterium* and *E. coli* auxotrophs at a reduced dilution rate. Following inoculation, the reactors were operated isochorically for 3.5 days before 5 days of continuous culture at a dilution rate of 0.075 hr^{-1}. Panels are labeled with the gene deleted from the *E. coli* strain. Cell densities were quantified every 24 hours and the $\log_{10}$CFU/ml for the co-cultured and monoculture bacteria are shown. *E. coli* cell densities are shown in red and *B. megaterium* cell densities are shown in blue. Monocultures are represented by dotted lines and cocultures are represented by solid lines. Each experiment was run in duplicate. One representative biological replicate is shown.

Figure 6. Growth of auxotrophs in mono- and co-culture on semi-solid M9+ media with sucrose and glucose. (Left) Schematic of mono-cultures and co-cultures inoculation on assay plates. (Right) Photographs taken after growth at 37 °C for 72 h. In photographs: (Top row) *E. coli* mono-cultures.

(Middle row) Co-culture of E. coli and B. megaterium. (Bottom row) mono-cultures of *B. megaterium*. *E. coli* strains are labeled along the top of each image and B. megaterium labeled at the bottom.