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BIOTECHNOLOGY

Balancing Trade-Offs Imposed by Growth Media and Mass Spectrometry for Bacterial Exometabolomics

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ABSTRACT The bacterial exometabolome consists of a vast array of specialized metabolites, many of which are only produced in response to specific environmental stimuli. For this reason, it is desirable to control the extracellular environment with a defined growth medium composed of pure ingredients. However, complex (undefined) media are expected to support the robust growth of a greater variety of microorganisms than defined media. Here, we investigate the trade-offs inherent to a range of complex and defined solid media for the growth of soil microorganisms, production of specialized metabolites, and detection of these compounds using direct infusion mass spectrometry. We find that complex media support growth of more soil microorganisms, as well as allowing for the detection of more previously discovered natural products as a fraction of total *m/z* features detected in each sample. However, the use of complex media often caused mass spectrometer injection failures and poor-quality mass spectra, which in some cases resulted in over a quarter of samples being removed from analysis. Defined media, while more limiting in growth, generated higher quality spectra and yielded more *m/z* features after background subtraction. These results inform future exometabolomic experiments requiring a medium that supports the robust growth of many soil microorganisms.

IMPORTANCE Bacteria are capable of producing and secreting a rich diversity of specialized metabolites. Yet, much of their exometabolome remains hidden due to challenges associated with eliciting specialized metabolite production, labor-intensive sample preparation, and time-consuming analysis techniques. Using our versatile three-dimensional (3D)-printed culturing platform, SubTap, we demonstrate that rapid exometabolomic data collection from a diverse set of environmental bacteria is feasible. We optimized our platform by surveying *Streptomyces* isolated from soil on a variety of media types to assess viability, degree of specialized metabolite production, and compatibility with downstream LESA-DIMS analysis. Ultimately, this will enable data-rich experimentation, allowing for a better understanding of bacterial exometabolomes.

KEYWORDS specialized metabolites, natural products, DIMS, LESA-MS, cultivation conditions, exometabolome, *Streptomyces*

The immense diversity of molecules that bacteria produce makes it challenging to study their secreted specialized metabolites, or exometabolome, in a holistic manner. Exometabolome composition depends on a variety of factors, including nutrient abundance, cultivation conditions, and the presence of competitors (1, 2). In particular, soil bacteria are known to secrete an array of specialized metabolites whose production often depends on environmental stimuli (3). Many studies have focused on the production of individual metabolites, such as specific antibiotic compounds (4). Yet, studying the entire breadth of molecules

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comprising the exometabolome poses inherent technical and practical difficulties. One of these challenges is the choice of growth medium, which affects both an organism's ability to grow robustly and produce specialized metabolites. Furthermore, medium presents an auxiliary signal that may interfere with detection of the exometabolome (5, 6). Because it is often only practical to use a single growth medium for experiments, it is critical to choose a medium that balances multiple considerations.

Growth media optimization has largely focused on eliciting or increasing production of targeted compounds (4, 7, 8). Though it is known that increased complexity of media sometimes leads to an increased diversity of specialized metabolites (9), less is known about optimizing media toward this goal. All bacteria require sources of carbon, nitrogen, inorganic phosphate, and trace metals to grow (10). Both the ability to grow and produce specialized metabolites are dictated by how metabolic precursors are processed through the metabolic network. Complex media typically provide bacteria with a richer array of metabolic building blocks, which can support growth and elicit production of a greater abundance and diversity of specialized metabolites by reducing the energy required to synthesize metabolic precursors *de novo* (11).

Although complex media might be advantageous for specialized metabolite production, it can interfere with the detection of these compounds if the growth matrix is not removed prior to analysis via mass spectrometry. Direct infusion mass spectrometry (DIMS) is a common method used for untargeted metabolomics (12, 13), whereby a sample is analyzed directly by mass spectrometry without a prior separation step (e.g., liquid chromatography). The absence of a separation step may lead to the inability to detect some molecules within a sample due partly to ion competition between the different analytes and the media components present in the sample matrix (i.e., growth medium) (14). The ideal sample matrix for maximizing analyte detection would be low in salts and other nonvolatile compounds, while containing only well-characterized organic components in order to simplify background subtraction in downstream analyses. However, the minimalistic composition of defined media may constrain the growth and production of specialized metabolites by bacteria. This is particularly problematic in microbiome studies when multiple bacteria are required to grow on the same medium.

Since the number of potential growth media is vast, a heuristic procedure must be employed to narrow the possibilities. In this study, we set out to identify a solid medium that would support the robust growth of soil bacteria and allow detection of secreted specialized metabolites with DIMS. We focused on choosing a growth medium for bacteria belonging to the genus *Streptomyces*, which are well-known for the diversity of specialized metabolites they produce (1, 5, 15, 16). We sought a medium that would both elicit the production of specialized metabolites and facilitate DIMS detection, two potentially competing objectives. To accomplish this task in high-throughput, we relied on the SubTap growth platform we previously developed for DIMS (13). This permitted characterization of trade-offs between complex and defined media in a manner that could be applied to rationalize the selection of growth media for future studies of the exometabolome.

RESULTS AND DISCUSSION

Because the number of possible combinations of media components is endless, we began with a top-down approach. First, we studied many media for their ability to support growth of the most microorganisms in different soil samples. We then narrowed the number of organisms and media to look at the growth of unique bacteria in greater detail. This was followed by comparison of a smaller subset of media and bacteria from the genus *Streptomyces* to investigate their exometabolome with DIMS.

Some soil microorganisms can grow on minimalistic defined media. "The great plate count anomaly" is a well-known phenomenon, in which only a small fraction of soil microorganisms can be easily grown on standard laboratory growth media (17–20). Determining the concentration of colony forming units (CFU) in distinct soil samples across different media offers a simple means of comparing media for their general ability to support bacterial growth. To this end, we selected a diverse collection of 21 solid

TABLE 1 Complex and defined media used in this study

Media type	Component(s)	Product no.
NB	8 g/L nutrient broth	RPI 50-488-846
ISP1	5 g/L tryptone	BD 211705
	3 g/L yeast extract	Fisher BP1422
ISP2	10 g/L malt extract	BD 218630
	4 g/L yeast extract	Fisher BP1422
	4 g/L glucose	Fisher D16
CAA	2 g/L acid hydrolyzed casein	Fisher BP1424
G-N-P-T	55.5 mM glucose	Fisher D16
	10 mM $(NH_4)_2SO_4$	Fisher BP212R
	25 mM potassium phosphate buffer (pH = 7.4)	Fisher BP362 Fisher BP363
	1 mM $MgSO_4$	Fisher M65
	100 nM $CaCl_2$	Sigma C4901
	100 nM NaCl	Sigma S9888
	10 nM $FeSO_4$	Sigma 215422
	1 nM $ZnSO_4$	Sigma 221376
	1 nM $MnSO_4$	Sigma M7634
	1 nM $CuSO_4$	Fisher BP346
S-N-P-T	5 g/L starch All compounds inG-N-P-T except glucose	Sigma S9765
AA-P	5.6 g/L amino acid mix	Teknova C0704
	25 mM potassium phosphate buffer (pH = 7.4)	Fisher BP362 Fisher BP363

media spanning minimal defined media to complex media (Table 1; Fig. 1). Our defined media were comprised of combinations of a carbon source (glucose or starch, G or S), nitrogen source (ammonium sulfate, N), inorganic phosphate source (phosphate buffer, P), and trace elements (T). Because agar contains impurities that may support growth, we tested both agar and highly purified noble agar as our solidifying agent. We were also interested in how the inclusion of amino acids affected overall growth and included them either as a complex mixture (casamino acids, CAA) or a defined mixture of all amino acids at known proportions (AA).

Based on the concentration of colony-forming microorganisms per medium, defined media supported the growth of fewer CFU than complex media (Fig. 1) (two-way ANOVA, $P = 5e\text{-}14$). Surprisingly, agar alone only caused a roughly 10-fold reduction in growth compared to any type of complex media (i.e., those above the green horizontal bar in Fig. 1) (two-way ANOVA, $P = 0.01$), and this was largely unaffected by supplementation with glucose, starch, nitrogen, phosphate, trace elements, or amino acids. In contrast, noble agar resulted in about a 1,000-fold reduction in growth compared with complex media (two-way ANOVA, $P = 0.004$), which was partly ameliorated by supplementation with other components. Supplementation of agar or noble agar with amino acids did not support the growth of as many CFU as supplementation with CAA (two-way ANOVA, $P = 0.005$). Taken together, these results are consistent with the expectation that complex media supports the growth of more soil microorganisms. As this experiment was intended to assess growth of *any* culturable bacteria present in a given soil sample, additional data elucidating the identity of observed colonies was not collected, although it is likely that different media supported the growth of different species.

Complex media support more robust growth than defined media. Although colony counts provide an aggregate overview of culturability, individual species of bacteria are not present at equal proportions in a single soil sample, and domination of a few key community members likely drives aggregate trends. We, therefore, isolated a group of genetically distinct bacteria from a single soil sample (Fig. 2) and compared their individual growth characteristics on six complex and 10 defined media types. As shown in Fig. 3, nearly all of the isolates grew in complex media (93% to 100%; Fig. 3A), and growth was observable within 24 h for the majority of isolates (mean time to visible growth of 15 h; Fig. 3B). Defined media with agar supported less growth (59% to 95%) and slower growth (mean time to visible growth of 25 h) than complex media. As expected, defined media with

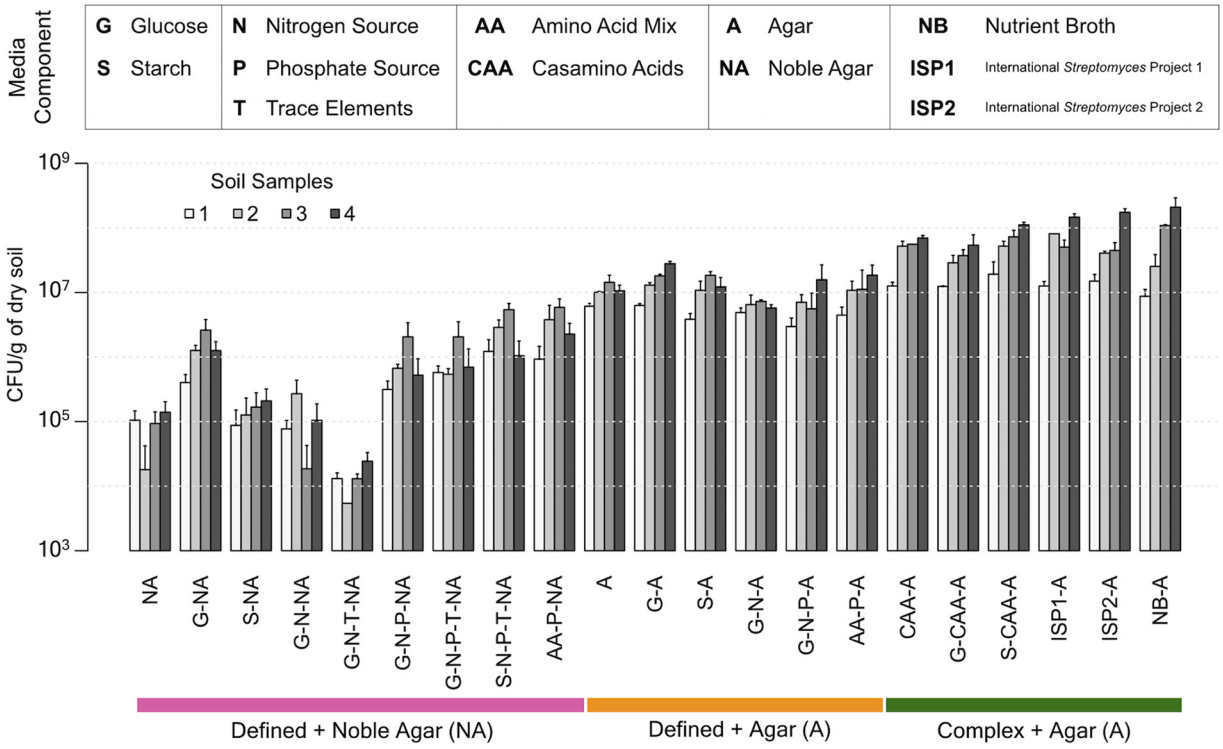

FIG 1 Culturable bacteria from four soil samples grown on various solid media. Complex media with agar supported the growth of approximately one order of magnitude more CFU than defined media. Similarly, agar supported more growth than noble agar as a solidifying agent, presumably due to impurities that are removed during the noble agar purification process. Error bars indicate the standard deviation of three replicates.

noble agar supported the least growth (7% to 50%) and slowest growth (mean time to visible growth of 29 h). Overall, these trends mirrored that of the original soil samples with the exception of inorganic phosphate addition, which greatly improved the fraction of bacterial isolates that grew and decreased the time to visible growth.

A substantial fraction (22 of 59) of our soil isolate collection was originally isolated on media designed to select for Actinomycetes, including *Streptomyces* species. Specialized metabolite production among *Streptomyces* is often synchronized with the sporulation phase of their life cycle (5). Sporulation causes a morphological change that can be identified during growth (Fig. 2A). Overall, complex media supported faster visibly identifiable sporulation (Fig. 3C and D). While CAA alone supported a greater number of isolates with visibly identifiable sporulation (91%), amino acid defined medium did not (5%). Addition of glucose to CAA greatly suppressed sporulation (from 91% to 32%), probably because sporulation is often triggered by starvation (5, 21, 22). These results indicated an overall advantage of complex media for growth and sporulation *of Streptomyces*.

Media differ considerably in their utility for mass spectrometry. Injection of extracts from complex media into mass spectrometers may lead to decreased detection of microbial compounds of interest due to interference from medium components and signal suppression by salts (23). Therefore, the "ideal" media for DIMS would be the absence of media, which could only be approximated through time-consuming separation procedures (e.g., liquid chromatography) that introduce their own analytical biases. To circumvent these downsides, we sought a medium that would minimize interference in liquid extraction surface analysis (LESA)-based DIMS experiments. We selected a subset of seven media to test based on the results of our growth experiments, including three defined and four complex media. A visual summary of observed growth for this subset of media can be found on the right side of Fig. 2. *Streptomyces* from our isolate collection were cultured on each medium in up to four replicates using the SubTap platform that we previously developed for characterizing the exometabolome in high-throughput (see Fig. S1) (13). Mass spectra were acquired in

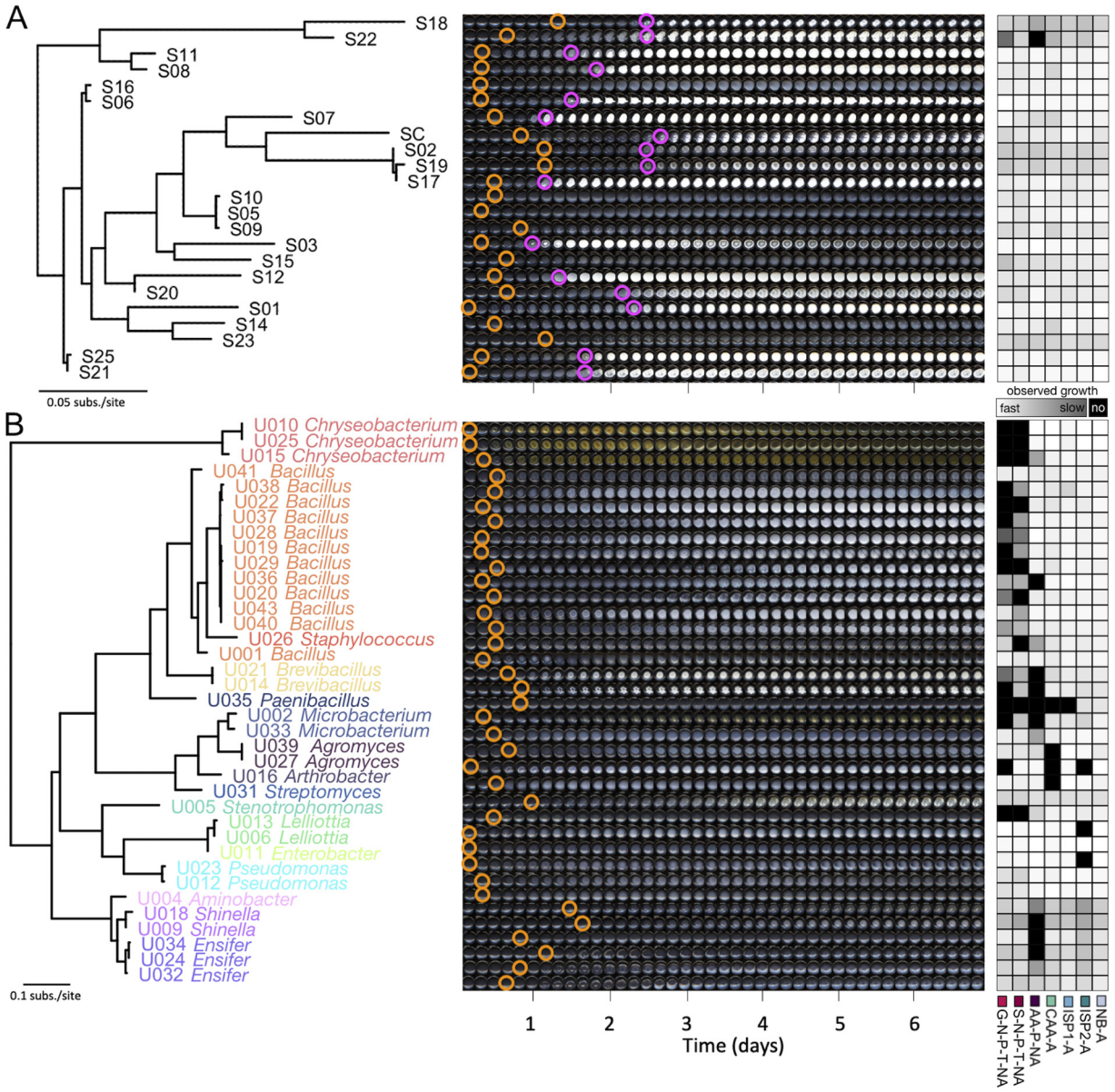

FIG 2 Phylogenetic trees, growth tracking images and speed of observed growth of bacterial isolates used in this study. (A) Maximum likelihood tree constructed from the *rpoB* gene of *Streptomyces* strains. (B) Maximum likelihood tree constructed from the 16S rRNA gene of all other soil isolates, colored based on taxonomic classification (genus level). The center of each panel shows time lapse well images of growth on NB-A at 28°C. Brightness and contrast of all images were increased uniformly by 40% to make growth and sporulation more obvious. Orange circles indicate first visible growth; magenta circles indicate first visible sporulation (when present). The right side of each panel depicts speed of observed growth on several media types used in this study.

positive ion mode using the spectral stitching method (24) that merges overlapping selected ion monitoring (SIM) windows for improved resolution and sensitivity.

First, we assessed the overall quality of data collected on each type of medium. This included flagging injection failures and identifying spectra that contained mass-to-charge (*m/z*) windows with missing or poor-quality data. Electrospray destabilization prior to mass spectrometry data acquisition was the primary culprit of injection failures. Notably, the number of mass spectrometer injections that passed each of our quality filters differed considerably from medium-to-medium. Three of the four complex media had high rates of injection failure (6% to 24%), while CAA-A and all three defined media had no failed injections (Fig. 4A). For injections that were successful, we identified any spectra that contained SIM windows where no *m/z* features were recorded (e.g., "empty windows"), as well as windows where the total signal-to-noise ratio was less than 1% of mean window signal-to-noise ratio in a given plate

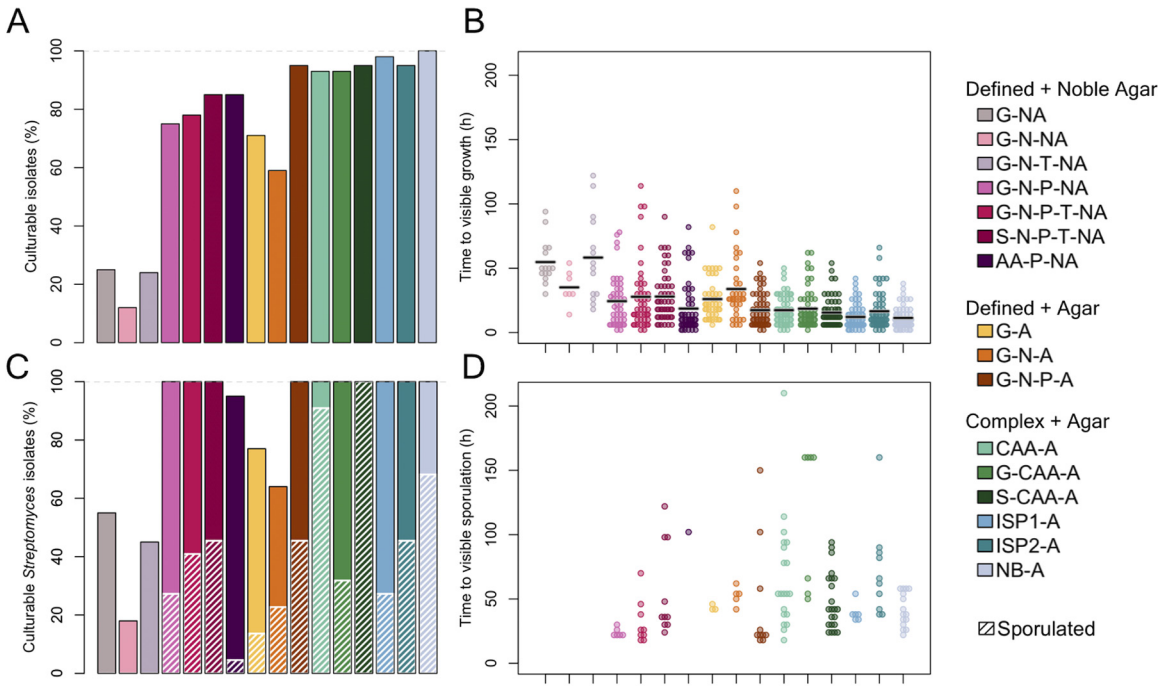

FIG 3 Characteristics of 59 soil bacterial isolates (including 22 *Streptomyces* isolates) grown on various solid media. (A) Complex media generally supported more growth than defined media. The inclusion of an inorganic phosphate source assisted growth in defined media. Note, all isolates were required to grow on NB-A for inclusion in the experiment. (B) Of the strains that grew (points), complex media enabled faster time to visible growth than defined media. Black bars indicate median time to visible growth. (C) Of the 22 *Streptomyces* isolates, only a fraction that grew also sporulated within 10 days, with the exception of S-CAA-A where all isolates both grew and sporulated. Crosshatching indicates percentage of *Streptomyces* isolates that sporulated. (D) Time of first visible sporulation for *Streptomyces* isolates varied considerably from strain-to-strain.

(i.e., low SNR windows). Complex media suffered from a greater number of injections that contained compromised windows (5% to 34%) than defined media (0% to 22%). Of the media tested, G-N-P-T-NA and S-N-P-T-NA had the fewest injections flagged for poor quality data (0% to 3%). Finally, although many injections resulted in spectra with empty or low signal-to-noise ratio windows, a subset of these had good quality data in the rest of the spectrum. For this reason, a successful injection was removed from subsequent analysis only if it had fewer than 500 total *m/z* features (Fig. 4A). This quality filtering approach generally resulted in removal of a greater fraction of injections from complex media (7% to 26%) than defined media (0% to 14%). The main exception was AA-P-NA defined medium, which performed similarly to the complex media that were tested in terms of number of injections that were removed from analysis. We suspect that the relatively high total amino acid concentration in this medium (nearly 50 mM), which was prepared according to the manufacturer's instructions, contributed to the high rate of compromised injections. It should be noted that the number of injections per isolate used in this study (four injections) surpassed the number required to have a 99% chance of observing at least one successful injection per isolate on each media type, assuming independence among injections ($\geq$1 for G-N-T-P-NA; $\geq$2 for S-N-T-P-NA and ISP2-A; $\geq$3 for AA-P-NA, CAA-A, ISP1-A; and $\geq$ 4 for NB-A).

Following our quality filtering step, the raw number of *m/z* features that were detected in each well were tallied. All three defined media tested produced spectra that contained a greater number of *m/z* features than complex media postfiltering (Fig. 4B). Processing of our data included a background subtraction step to remove any peaks present in the medium alone. For each plate, an *m/z* feature was subtracted if it was observed in any of the negative-control (media only) wells. The fraction of *m/z* features attributable to media background varied greatly from medium-to-medium and well-to-well (Fig. 4C). In particular, ISP2-A medium was responsible for the majority (90%) of *m/z* features in each of its wells, implying relatively few bacteria-produced compounds were detected on this medium. In contrast, S-N-P-T-NA

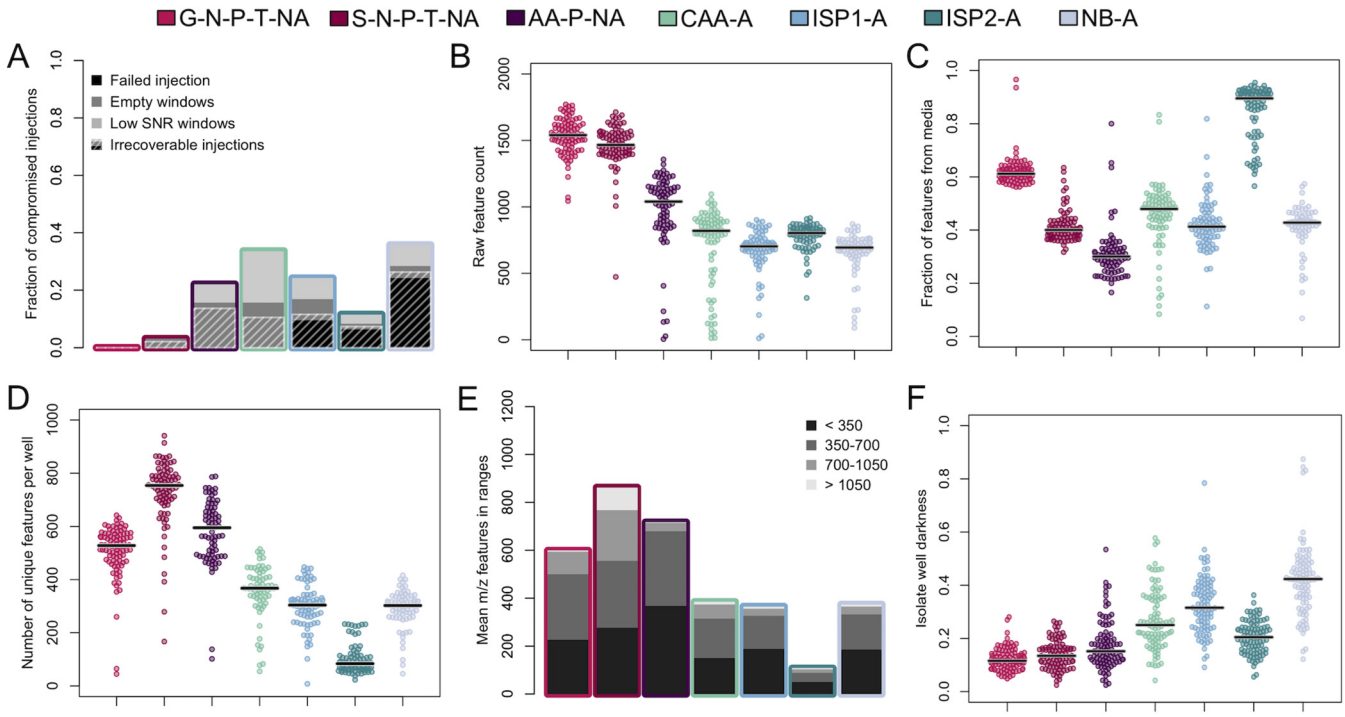

FIG 4 Summary of LESA-MS analysis of *Streptomyces* grown on complex and defined media. (A) Compromised injections occurred frequently with some media. Crosshatching indicates the fraction of injections that were removed from further analysis. (B) Prior to background subtraction, defined media have more detectable features than complex media. Each point represents a single well. (C) Media-related features comprised a large fraction of total *m/z* features detected in some media. (D) After removing features attributable to the media (4C) and correcting for multiple adducts, defined media contained more *m/z* features than complex media. (E) Most wells had few features detected above 700 *m/z*, with the exception of S-N-P-T-NA. (F) Complex media generally resulted in darker SubTap analysis plate wells than defined media. Horizontal black bars indicate median values.

had a large number of raw *m/z* features (Fig. 4B) and a relatively low fraction of features attributable to the medium (Fig. 4C).

To accurately enumerate unique specialized metabolites produced by our isolates, we collapsed common adducts (H^+, Na^+, K^+) in each spectrum after background subtraction. The greatest number of *m/z* features remained in wells belonging to defined media (Fig. 4D). This was especially true for S-N-P-T-NA wells, which often had more than twice as many *m/z* features as complex media wells after adduct collapsing and background subtraction. Most of the detected *m/z* features were in the range of 70 to 700 *m/z* (Fig. 4E). S-N-P-T-NA had more features above 700 *m/z* relative to the other media types (one-sided Mann-Whitney test, p-adj = 6e-7). Frequency of detection of high *m/z* features has been previously used as a surrogate for specialized metabolite production in other studies because some natural products have high molecular weights (9).

Media differ considerably in selectivity for Streptomyces natural products. We sought a medium that would permit both production and detection of secreted specialized metabolites. Although S-N-P-T-NA had the most *m/z* features remaining after background subtraction (Fig. 4D), it was unknown whether these features were enriched for specialized metabolites. Because many natural products are pigmented (25), measuring well darkness may provide an orthogonal metric to compare specialized metabolite production. Thus, we measured the darkness of wells in the SubTap analysis plates (Fig. S1) as a proxy for specialized metabolite production. It is worth emphasizing that the SubTap platform physically separates biomass from specialized metabolites because it contains a 0.2-μm membrane between the context plate, where bacterial growth occurs, and the analysis plate, where secreted specialized metabolites have diffused during growth. After background brightness correction for each medium by itself, complex media resulted in substantially darker substrates than defined media (Fig. 4F). If we assume well darkness is related to increased specialized metabolite production, this presents a potential trade-off between the merits of complex media for specialized metabolite production and the benefits of defined media for

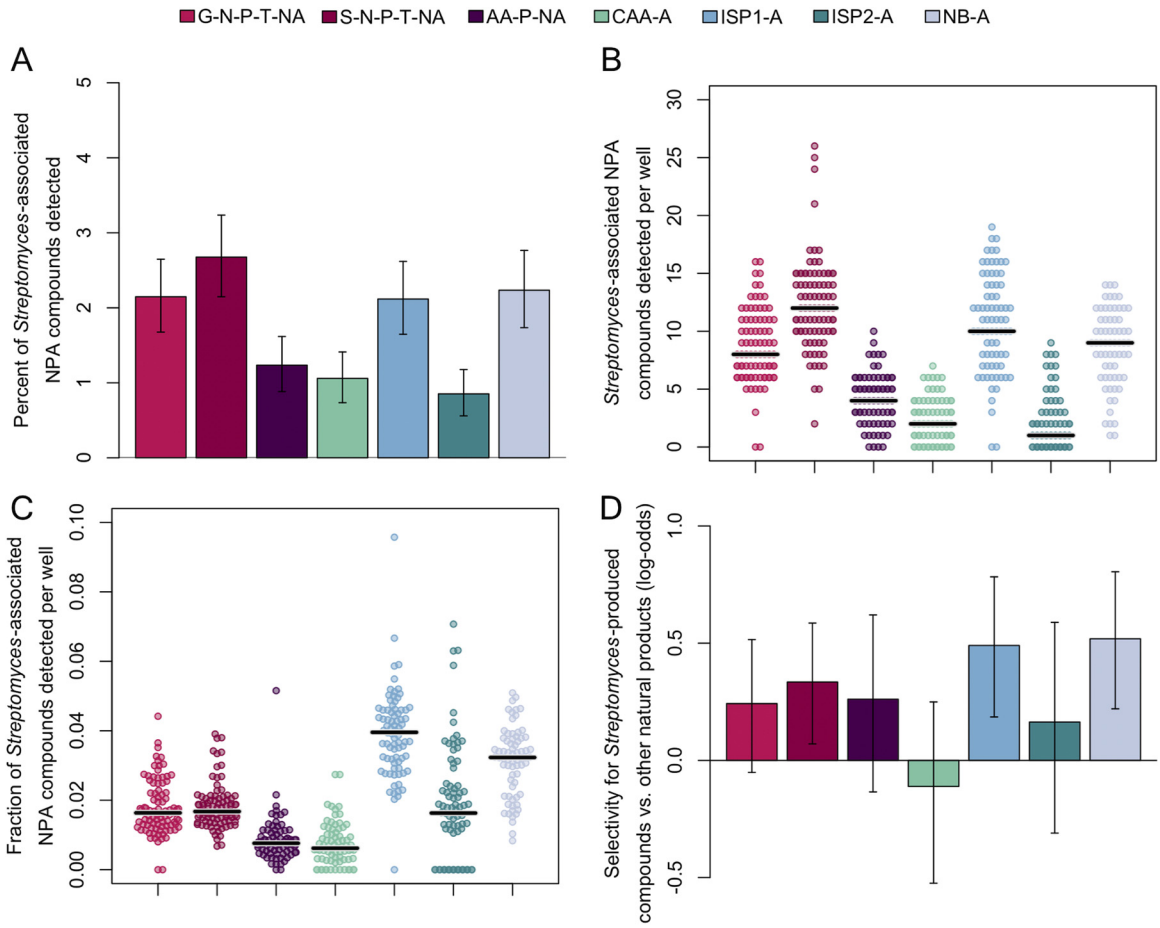

FIG 5 Detection of compounds found in Natural Products Atlas (NPA), by media type. (A) S-N-P-T-NA, NB-A, G-N-P-T-NA, and ISP1-A had the highest coverage of *Streptomyces*-produced compounds found in the Natural Products Atlas detected in each plate, as well as (B) the highest number of these compounds detected per well. (C) ISP1-A and NB-A had the highest fraction of *Streptomyces*-produced compounds detected in each well, as a function of total number of *m/z* features detected per well (Fig. 4D). Horizontal black bars indicate median values. (D) ISP1-A and NB-A displayed the greatest selectivity for matching *Streptomyces*-produced compounds over other compounds found in the Natural Products Atlas. Error bars in (A) and (D) depict the 95% confidence interval estimated with bootstrapping.

mass spectrometry. However, this assumption may be incorrect because well darkness could be driven by a subset of compounds that are pigmented and does not distinguish diversity of compounds from abundance of production. This is consistent with the observation that although S-N-T-P-NA does not have dark wells, it has the greatest number of unique *m/z* features observed via MS—highlighting an advantage of using mass spectrometry for screening production of specialized metabolites.

To further delineate this potential trade-off, we used the Natural Products Atlas (26) to obtain a list of 4,830 known compounds produced by members of the genus *Streptomyces*, 3,400 of which had unique masses in our mass range. We searched for the exact masses of their H^+, Na^+, and K^+ pseudomolecular adducts (z = 1) in each well across our media. Because MS1 data are insufficient for confident identifications (27), we required the presence of multiple adducts of a compound to increase the confidence of positive matches in our search results. We reasoned that media associated with exometabolomes containing a greater fraction of known compounds better balance the trade-off between microbial growth and spectrum quality in mass spectrometry.

Based on fraction of known *Streptomyces*-produced compounds identified across all samples (Fig. 5A), S-N-P-T-NA outperformed the other media with 2.7%, followed by NB-A with 2.2%, ISP1-A and G-N-P-T-NA with 2.1%, although these differences were not statistically significant. AA-P-NA and CAA-A both performed poorly with 1.2% and 1.1% identified, respectively, while ISP2-A only matched 0.9% of the *Streptomyces*-produced compounds in

the Natural Products Atlas. Although this measure suggests that there is a greater diversity of previously discovered specialized metabolites produced on S-N-P-T-NA, more wells were excluded from analysis on complex media (especially NB-A), inherently limiting the possible search space. On a well-by-well basis, the highest number of the Natural Products Atlas matches were detected on S-N-P-T-NA (Fig. 5B) (one-sided Mann-Whitney test, p-adj = 0.045). However, as a fraction of total observed *m/z* features per well, ISP1-A performed significantly better than any of the other media types (Fig. 5C) (one-sided Mann-Whitney test, p-adj = 2e-4). Finally, we quantified the selectivity for known compounds produced by *Streptomyces* over compounds associated with all other organisms in the Natural Products Atlas (Fig. 5D). Within each media type, only S-N-P-T-NA, ISP1-A, and NB-A displayed selectivity for *Streptomyces*-produced compounds that was statistically significant (p-adj = 0.049, 0.007, and 0.003, respectively). Taken together, our results suggest that some complex media elicit greater specialized metabolite production but some defined media are preferable for DIMS.

It was previously shown that phylogenetic distance is correlated with the metabolome (28). Thus, to validate our Natural Products Atlas results, we sought to link our specialized metabolite data with the genetic relatedness of our *Streptomyces* isolates. Many matches to the Natural Products Atlas displayed a clustering of presence/absence patterns on a maximum likelihood phylogenetic tree constructed from the *rpoB* gene (Fig. S3A). We quantified the overall degree of clustering by constructing trees from the observed presence/absence pattern on each medium and quantifying the topological distance to the phylogenetic tree. Media with higher selectivity for *Streptomyces*-produced compounds also tended to display lower tree distances (Fig. S3B), corroborating our Natural Products Atlas search results.

Balancing trade-offs in exometabolomics. In this study, we characterized the effect of media on variables of importance for exometabolomics. In general, we found that (i) complex media support better growth of soil microorganisms than defined media, (ii) complex media result in a greater number of failed injections and poorer quality mass spectra than defined media, (iii) complex media yield fewer *m/z* features after background subtraction than defined media, and (iv) complex media likely elicit greater specialized metabolite production than defined media. These findings present a trade-off between microbial growth and detection of secreted specialized metabolites.

Our results have a number of limitations. First, we did not vary the levels of media components, nor did we test many other combinations of media ingredients that are possible. Second, we only analyzed growth and sporulation over 10 days for practical reasons, although we suspect the results would not change considerably because most growth was observed long before this time point. Third, we used a single extraction solvent (70% methanol/0.1% formic acid) for our LESA-MS method, which likely would not have extracted all possible compounds present in our SubTap plates with equal probability. Alternative extraction solvents were not studied systematically. Fourth, we only analyzed the replicates that passed our quality filters, which effectively incorporated failed injections indirectly into our measures of observed *m/z* features because failed wells contain no features. Fifth, we used compounds in the Natural Products Atlas as a proxy for specialized metabolite detection. It is possible that these compounds have a bias associated with their inclusion in the Natural Products Atlas, such as being more likely to be produced on complex media. Similarly, it is possible that compounds in the Natural Products Atlas marked as originating from non-*Streptomyces* can be produced by members of the *Streptomyces* and should have been counted as such in the calculation of selectivity. In addition, almost all of our *Streptomyces* were absent from the Natural Products Atlas, so we have no prior evidence they can produce the *Streptomyces* compounds in the Natural Products Atlas. Lastly, we did not explore the use of negative ion mode detection in our mass spectral analyses, and it was assumed that all compounds present in the Natural Products Atlas were capable of forming positively charged pseudomolecular adducts for subsequent detection in positive ion mode.

SubTap paired with LESA-DIMS analysis was designed for efficiency and affordability, allowing for the identification of patterns of compound production by comparing across many samples. While it is true that many of the issues that were encountered may have been overcome by alternative sample preparation or pre-mass analysis chromatographic steps, our

TABLE 2 Media used for initial isolation of the bacteria used in this study

Media type	Component(s)	Product no.
LBA	25 g/L Luria-Bertani broth	Fisher BP1426
NA-0.5% peptone	8 g/L nutrient broth	RPI 50-488-846
	5 g/L peptone	Fisher BP1420
YEA	3 g/L yeast extract	Fisher BP1422
MEA	30 g/L malt extract	BD 218630
EDM2, pH adjusted to 7.0 using NaOH	1 g/L starch	Sigma S9765
	15 mM KH_2PO_4	Fisher BP362
	35 mM NaCl	Sigma S9888
	0.5 mM $MgSO_4$	Fisher M65
	20 mM KNO_3	Sigma 221295

aim was to develop a workflow that would enable low-cost, high-throughput, first-pass screening with direct infusion. The number of injection failures and poor-quality spectra collected from wells with complex media partly preclude their use for high-throughput DIMS data acquisition. This resulted in over a quarter of the wells from NB-A being removed from analysis. Therefore, if an experimental design prohibits the inclusion of a high number ($n \geq 4$) of replicate wells on a plate, complex media may not be a viable option. On the other hand, defined media limit the number of microorganisms that can be cultured and likely the array of compounds they can produce. For organisms that *are* able to grow, however, as little as a single well is necessary to ensure successful data collection, enabling high-throughput screening experiments. As it is often only practical to use a single medium type in an experiment, these tradeoffs should be kept in mind when selecting a medium. If injection failures are a minimal concern, complex media that elicit specialized metabolite production (e.g., ISP1-A and NB-A) performed well. We prefer S-N-P-T-NA to avoid injection failures and obtain the greatest diversity of *m/z* features, although S-N-P-T-NA did not support the growth of some soil bacteria.

MATERIALS AND METHODS

Media composition. Recipes for rich and defined media used throughout our experiments can be found in Table 1. To better understand the contribution of impurities present in agar, media types were combined with either agar (Fisher BP1423) or noble agar (Research Products International 50-488-478) at the following concentrations: 0.5% for SubTap experiments, 1.5% for isolate selection, and 2% for all other experiments. All media were used as-is, without adjusting pH, unless noted otherwise.

Soil communities and individual soil isolates. Soil bacterial communities originated from four soil samples collected from locations at latitude and longitude coordinates <40.437686, −79.908732> (#1), <40.413835, −79.917433> (#2), <40.450958, −79.952528> (#3), and <40.431609, −79.963394> (#4). To create frozen soil community stocks, 1 g of each soil sample was mixed with 50 mL of ultrapure water and shaken with glass beads for 30 min. After filtering out coarse soil particulates using a paper filter, 20 mL of this solution was mixed with 30 mL of sterile glycerol for a final glycerol concentration of 60%. Aliquots were frozen at −80°C until use. Each aliquot was only thawed a single time, immediately before use, to standardize any loss in viability associated with freezing or thawing. Soil samples from each location were weighed after drying at room temperature for 7 days to determine dry weight.

Individual soil bacteria were isolated from the soil sample collected at location #4. Using a sterile pipette tip, 500 mg of soil was removed from the center bottom of the collected soil plug. The sample was serially diluted by 10-fold increments to 10^{-5}, and 100 μL of each dilution was plated in triplicate on Luria-Bertani agar, nutrient agar with 0.5% peptone, yeast extract agar, malt extract agar, and EDM2 (29) (Table 2). We added 50 mg/L cycloheximide to all media to suppress fungal growth. All media were prepared with and without soil extract by replacing half of the water volume by an equivalent volume of soil extract, resulting in 10 total media. Soil extract was prepared by mixing 1 kg of soil from location #4 with 2 L of water. The resulting slurry was coarse filtered to remove solids, followed by passage through a 0.2-μm filter to remove fine particulates and autoclaving to ensure sterility.

Soil dilutions (10^{-2} to 10^{-6} final dilution) were spread on individual agar plates of each media type in triplicate and incubated at 28°C for 7 to 14 days. Individual colonies that appeared unique based on morphological characteristics were picked with a sterile toothpick and pinned onto wells of a black 96-well plate prepared with their original growth medium. Each plate was inverted on an Epson Perfection V550 scanner, secured with packing tape to mitigate desiccation, and scanned every 4 h for 7 to 14 days at 28°C. Images were processed programmatically by clustering colonies based on time to visible growth, growth rate, pigmentation, opacity, and size. After visual inspection, a representative colony from each cluster was subsequently restreaked on its respective agar type and incubated at 28°C for 7 to 14 days. This process resulted in a set of isolates that appeared morphologically distinct, but DNA sequencing was still required to confirm uniqueness.

DNA extraction and sequencing. To lyse the cells, individual colonies were suspended in 100 μL of Tris-EDTA buffer and sonicated for 1 min at 100% amplitude with a QSonica Model 505 Sonicator with cup horn. Each suspension was centrifuged for 1 min at 14,000 r.c.f. and supernatant containing the genomic DNA was collected. To confirm uniqueness by sequencing, quantitative PCR (qPCR) was performed using primers internal to the *rpoB* gene (for *Streptomyces* isolates grown on EDM2: rpoB-F: 5′-CTCGACATCTACCGCAAGCT and rpoB-R: 5′-GACACCATCTGGCGCG) (29) or 16S rRNA gene (27F: 5′-AGAGTTTGATYMTGGCTCA and 1492R: 5′-GGTTACCTTGTTACGACTT). A total of 0.4 μL of each (10 μM) primer, 5.0 μL iTaq Supermix, 1 μL of DNA template, and 3.2 μL of sterile water were used for each reaction. Cycling conditions on an Applied Biosystems StepOnePlus real-time PCR system began with an initial denaturation of 98°C for 2 min, followed by 40 cycles of 98°C for 15 s, 64°C for 30 s, and 80°C for 30 s, with a final melt stage at 60°C to 95°C with 10-s steps at a ramp rate of 0.3°C per step. Amplicons were Sanger sequenced and chromatograms were reviewed for quality. The isolates' sequences were aligned to create maximum likelihood phylogenetic trees using the R package DECIPHER (30). Classification of 16S rRNA sequences was carried out with IDTAXA (31) using the SILVA training set at 60% confidence.

Growth profiling of soil communities and isolates. To test soil community growth on different media, each frozen aliquot was diluted 10-fold six times in 60% glycerol with 30 μL of each dilution plated in triplicate. All plates contained 50 μg/mL cycloheximide to prevent fungal growth. Plates were counted after incubating for 7 days at 28°C. For each sample/media combination, we counted the dilution with the fewest countable colonies (generally, $3 < x < 30$ colonies) to avoid possible growth inhibition from colonies in close proximity and interfering spreading phenotypes that were prevalent at higher concentrations.

To profile the growth of individual soil isolates, each isolate was spotted onto an opaque (black) 96-well plate containing a single solid medium type. Each plate was inverted on an Epson Perfection V550 scanner, secured with packing tape to minimize desiccation, and full-color scans were captured every 4 h for 10 days at 28°C to monitor growth and sporulation. In total, 59 isolates were profiled (22 *Streptomyces* and 37 other). Though these bacteria were originally isolated from soil on different media (Table 2), at a minimum, all isolates were required to grow on NB medium to be included. Fig. 2 was generated by cropping each scanned time point and arranging them by well to create movie strips depicting how each well changed over time.

Cultivation for mass spectrometry experiments. The 22 *Streptomyces* isolates were tested on a subset of media for analysis of specialized metabolite production and detection. Using the SubTap platform (13), each isolate was grown in a single well containing 120 μL media with 0.5% agar or noble agar on the culture plate, separated by a 0.2-μm polycarbonate track-etched (PCTE) membrane (GVS Group) from the analysis plate containing 30 μL of 0.5% noble agar without medium. Each isolate was grown in quadruplicate on every medium, except for S01 which was only grown in triplicate due to plate space limitations. *S. coelicolor* was included in the *Streptomyces* collection as a control strain. After 7 days of growth at 28°C, the top culture plate containing bacteria was removed and bottom analysis plate dehydrated, full-color scans were obtained using an Epson Perfection V550 scanner, and frozen until time of analysis. Analysis plate images were analyzed for average well color intensity (defined as the mean of red, blue and green channels) over a 10 pixel $\times$ 10 pixel area centered in each well.

DIMS data acquisition. Data acquisition was performed using a Velos Pro Orbitrap mass spectrometer (Thermo Fisher Scientific) paired with a Triversa Nanomate robot (Advion) programmed using ChipSoftX to perform liquid extraction surface analysis (LESA) direct infusion experiments with the SubTap platform. With the analysis plate cooled at 8°C, each well was extracted with 70% methanol/0.1% formic acid (aspirate 20 μL solvent, dispense 15 μL on the well, hold for 4 s, aspirate 8 μL). The extract was injected into the mass spectrometer using a nanoESI chip (Advion). Mass spectra were collected in positive ion mode only. Quality control (QC) injections containing tune mix (Agilent G1969-85000) were collected every 10 injections.

Mass spectrometry data were acquired as several overlapping mass-to-charge (*m/z*) windows that are "stitched" together to create a complete mass spectrum (24). This method of data collection increases the dynamic range and detection sensitivity compared with collecting DIMS data as a single (wide) *m/z* window. Data were collected as 200 *m/z* SIM (selected ion monitoring) windows that overlap by 30 *m/z*. To allow for nanoelectrospray stabilization, data acquisition started after 45 s of sample infusion into the MS. The parameters used were 100 K mass resolution at 200 *m/z*, an overall mass range of 70 to 2,000 *m/z* with 27 total *m/z* windows, 15 s per *m/z* window acquisition time, and an AGC target of 10^6. The acquisition time per injection was approximately 6 min.

MS data processing and analysis. For each plate, injection failures were manually removed and raw data files were processed using DIMSpy v1.3.0 (32) with nondefault parameters in process_scans (min_scans = 1, snr_thres = 1.0, ppm = 3.0, ringing_thresh = 0.04, min_fraction = 0.25, rsd_thres = none) and align_samples (ppm = 3.0). This resulted in a list of *m/z* features by signal-to-noise ratio. Further postprocessing and data analysis was performed using a custom R script available on GitHub (https://github.com/digitalwright/ExometabolomicsGrowthMedia). This included truncating the *m/z* range to 70 to 1,400, using QC peaks to calibrate spectra, removal of ^{13}C isotopic peaks, removal of any features that do not occur in a plate at least once with a signal-to-noise ratio of at least 10, removal of any injections that contained low total signal-to-noise ratio windows with less than 500 *m/z* features, removal of features associated with polyethylene glycol, and removal of peaks associated with media or QC components. All statistical analyses were carried out in R (v3.6) (33). Where noted, *P*-values were adjusted for multiple comparisons using the Bonferroni correction.

Comparison with compounds in Natural Products Atlas. The Natural Products Atlas (26) (v2020_06) was downloaded from https://www.npatlas.org/. For investigating compounds known to be produced by *Streptomyces* (i.e., the "*Streptomyces* set"), all (4,380) compounds associated with the genus *Streptomyces* were selected and further reduced to only unique masses within the relevant *m/z* range (70 to 1,400), resulting

in 3,400 unique exact masses. To count as a match, all spectra were searched for the occurrence of at least two of three possible adducts (H^+, Na^+, K^+) within a 7-ppm tolerance for each compound in the set.

To better understand selectivity for compounds produced by *Streptomyces*, the 8,725 compounds from the Natural Products Atlas that were not associated with and not identical to the exact masses of compounds known to be produced by *Streptomyces* were selected (i.e., the "non-*Streptomyces* set") and searched in the same manner as the *Streptomyces* set. Confidence intervals (95%) were estimated through bootstrap replication by drawing 10^7 random (paired) samples of the *Streptomyces* and non-*Streptomyces* sets from a Poisson distribution with the expected value for each medium determined in our experiment. Selectivity (log-odds) was then defined as:

$$\text{Selectivity} = \log\left(\frac{\textit{Streptomyces}\,\text{hits}/3400}{\text{non}-\textit{Streptomyces}\,\text{hits}/8725}\right)$$

The frequency of matches to *Streptomyces*-produced compounds found in the Natural Products Atlas, by media type, can be found in Fig. S2. To investigate how well the Natural Products Atlas compound hits found in each media type reflect the genetic relatedness of our *Streptomyces* isolates, an unweighted pair group method with arithmetic mean (UPGMA) tree was constructed for each media using a binary presence/absence matrix of Natural Products Atlas hits. This tree was compared with the *rpoB* tree (Fig. 2A; Fig. S3A), and clustering information distance between the trees was calculated using the TreeDist R package (34) (Fig. S3B). A graphical representation of the patterns of production of four compounds in the Natural Products Atlas is also shown in Fig. S3A. Further information about the compounds from the Natural Products Atlas putatively annotated (level 3) (27) in Fig. S2 and S3 can be found in Table S1.

Data availability. Sequences are available from GenBank under the accession numbers MN177030-MN177054, MN186620-MN186656, and OK033102-OK033103.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.6 MB.

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