

Beyond Agar: Gel Substrates with Improved Optical Clarity and Drug Efficiency and Reduced Autofluorescence for Microbial Growth Experiments

Philipp A. Jaeger,^a Cameron McElfresh,^b Lily R. Wong,^c Trey Ideker^a

Departments of Bioengineering and Medicine, University of California San Diego, La Jolla, California, USA^a; Nanoengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of California San Diego, La Jolla, California, USA^b; Bioengineering Program, University of Califor

Agar, a seaweed extract, has been the standard support matrix for microbial experiments for over a century. Recent developments in high-throughput genetic screens have created a need to reevaluate the suitability of agar for use as colony support, as modern robotic printing systems now routinely spot thousands of colonies within the area of a single microtiter plate. Identifying optimal biophysical, biochemical, and biological properties of the gel support matrix in these extreme experimental conditions is instrumental to achieving the best possible reproducibility and sensitivity. Here we systematically evaluate a range of gelling agents by using the yeast *Saccharomyces cerevisiae* as a model microbe. We find that carrageenan and Phytagel have superior optical clarity and reduced autofluorescence, crucial for high-resolution imaging and fluorescent reporter screens. Nutrient choice and use of refined Noble agar or pure agarose reduce the effective dose of numerous selective drugs by >50%, potentially enabling large cost savings in genetic screens. Using thousands of mutant yeast strains to compare colony growth between substrates, we found no evidence of significant growth or nutrient biases between gel substrates, indicating that researchers could freely pick and choose the optimal gel for their respective application and experimental condition.

ingle-cell organisms such as bacteria and yeasts have been used extensively to study genes and genome organization, proteins and protein interactions, biological pathways, and cellular structure and to address numerous other fundamental biological questions (1, 2). Many microbes, especially the species Escherichia coli and Saccharomyces cerevisiae, combine numerous beneficial properties that make them ideal model organisms: easy laboratory cultivation, easy genetic manipulation, short generation time, and safe handling. Microbial cultures can be maintained in liquid growth medium or on solid/semisolid gel substrates (3). Liquid colony maintenance allows the microbial cultures to expand rapidly and is used in screens that rely on optical density measurements to extract kinetic growth parameters or use downstream liquid-based assays such as flow cytometry or high-throughput microscopy (4-7). While throughput in liquid is generally bound to a maximum of 96 or 384 samples per plate, solid-substrate colony maintenance has the advantage that individual cell clones can be readily separated and that colony parameters such as color, shape, structure, and size can be extracted easily using digital image acquisition (8-10). Additionally, advances in robotic pinning devices allow for much higher throughput on solid substrate, with close to 25,000 colonies possible on a single microtiter footprintsized gel plate (11). This much higher density has led to solidsubstrate screening being the method of choice for many current high-throughput applications (12–14).

Historically, agar has been the predominant gelling agent in microbial research. Agar is a mixture of polysaccharides from the cell wall of the marine red algae *Rhodophyta*, where it provides flexibility and strength (15). The Japanese innkeeper Minoya Tarozaemon discovered agar in 1658, when a seaweed jelly dish accidentally froze when left out overnight and subsequently dried to white powder in the morning sun. He observed that this powder could be redissolved in warm water and led to a clearer jelly than before (16). Fannie and Walther Hesse, assistant to the famous

German microbiologist Robert Koch, introduced the use of agar to microbial research in 1882 after realizing the superior gelling qualities of agar over the previously used gelatin (17). Agar exhibits hysteresis, reflected in its ability to gel (32° C to 40° C) and melt (~85°C) at very different temperatures, making it much more suitable for microbial research than the low-melting-point gelatin (25° C to 30° C) (16). Additionally, agar is indigestible by most organisms.

Thus, Bacto agar (a proprietary modified agar with "optimal" magnesium, calcium, iron, and copper concentrations) has become the *de facto* standard gelling medium. However, Bacto agar's opaque gel appearance and batch variability call into question whether agar is indeed the optimal gel matrix for state-of-the-art high-throughput screening techniques. Additionally, agar is not necessarily readily compatible with all medium formulations (18). Finally, laboratories working with solid medium screens routinely consume thousands of agar plates in a single experiment. It may thus be worthwhile to identify suitable, more cost-effective gelling agents with optical and growth properties that are identical to or

Received 22 April 2015 Accepted 7 June 2015 Accepted manuscript posted online 12 June 2015 Citation Jaeger PA, McElfresh C, Wong LR, Ideker T. 2015. Beyond agar: gel substrates with improved optical clarity and drug efficiency and reduced autofluorescence for microbial growth experiments. Appl Environ Microbiol 81:5639–5649. doi:10.1128/AEM.01327-15. Editor: S.-J. Liu Address correspondence to Trey Ideker, tideker@ucsd.edu. Supplemental material for this article may be found at http://dx.doi.org/10.1128 /AEM.01327-15. Copyright © 2015, American Society for Microbiology. All Rights Reserved. doi:10.1128/AEM.01327-15 better than those of standard agar. In this regard, agar is certainly not the only gel-forming substance. Other seaweed-based gelling agents include alginic acid sodium salts and carrageenans, gellan gum (Phytagel/Gelrite), which is produced by bacteria, and various bean and seed extracts, which also have gel-forming properties (i.e., guar gum). However, a controlled parallel comparison of different gelling agents for their ability to serve as high-throughput microbial substrates is lacking.

Here, we systematically evaluate the properties of potential replacements for Bacto agar for use in microbial experiments. A number of gel-forming substances are considered (Table 1). Agarose is agar that has been purified to remove agaropectin (a poorly defined, complex polysaccharide with sulfate or pyruvate side groups) (16). It has been used as a microbial substrate (19-22) but is much more commonly used as a matrix for gel electrophoresis (23). Noble agar is a bleached and washed derivative of agar, resulting in a whiter gel (24), and is occasionally used as a growth substrate (25, 26). Carrageenan is a ubiquitous emulsifying and gelling agent used extensively in food science and pharmaceutical research (27) and has been investigated as a substrate or immobilizer for yeast and microbial cells (28, 29). Phytagel forms very hard and clear gels that are widely used in plant sciences to study root development and as a seedling growth substrate (30). It is produced in a controlled fermentation process (31, 32). Phytagel has found use as a microbial growth medium (33), especially for thermophilic microbes (34). Alginic acid has numerous industrial applications as a gelling agent and thickener, in culinary arts, and in pharmaceutical sciences (35). It is widely used to encapsulate and immobilize cells (36, 37) and has been explored for use in protoplast culture (38, 39). Guar gum has many industrial and culinary applications as a thickener, emulsifier, and coating substance (40) and has been suggested as a microbial growth substrate (41). Gelatin has many industrial and culinary applications (42) and was used as microbial growth medium before the introduction of agar (43). It still finds use in culturing certain microbes, for example, to test for external protease activity (44). Very recently, bacterial cellulose has been suggested as a promising microbial cell culture substrate (45); however, due to the lack of commercial availability, we did not test this gelling agent.

MATERIALS AND METHODS

Gel preparation, selection markers, and media. The following gelling reagents were used: agarose (20-102, lot LF45120012; Genesee Scientific, San Diego, CA), Bacto agar (214040, lot 4202919; BD Biosciences, San Jose, CA), carrageenan (C1013, lot SLBK3896V; Sigma-Aldrich, St. Louis, MO), Noble agar (A5431, lot SLBJ3882V; Sigma-Aldrich), Phytagel (P8169, lot SLBK1372V; Sigma-Aldrich), alginic acid (A0682, lot O81M1093V; Sigma-Aldrich), and guar gum (G4129, lot SLBH5231V; Sigma-Aldrich). Supplemental reagents and media were Bacto yeast extract (212720; BD Biosciences), Bacto peptone (211820; BD Biosciences), magnesium sulfate (M7506; Sigma-Aldrich), Difco dextrose/glucose (215520; BD Biosciences), Difco yeast nitrogen base without amino acids (291920; BD Biosciences), and Difco yeast nitrogen base without amino acids and ammonium sulfate (233520; BD Biosciences). Synthetic complete medium (SC) and SC dropout medium were prepared by following standard procedures using amino acids from Sigma-Aldrich. If indicated, selective pressure was maintained using Geneticin (G418; KSE Scientific, Durham, NC), nourseothricin (clonNAT; Werner BioAgents, Jena, Germany), S-(2-aminoethyl)-L-cysteine hydrochloride (S-AEC) (A2636; Sigma-Aldrich), or L-(+)-(S)-canavanine (Can) (C9758; Sigma-Aldrich) at the indicated concentrations. Gelling, supplemental, and medium reagents were mixed in double-distilled water (ddH2O) and autoclaved for

| TABLE 1 Descr | iption of the different gelling | agents tested in this study ^{u} | | | | |
|-------------------------------|---------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|------------|------------|--------------------------------------------------------------|
| Gelling | | | | Setting | Melting | |
| agent | Synonym(s) | Components | Source | point (°C) | point (°C) | Comment(s) |
| Agarose | | Repeating unit of agarobiose: a disaccharide made up of D-calartose and 3.6-anhydror-1-calartonyrranose | Red algae (<i>Gelidium</i> spp., Gracilaria snn) | ~ 36 | ~ 88 | Agar without agaropectin |
| Bacto agar | Kanten, agar-agar | Agarose (70%) plus agaropectin (30%) | Graciaria spp.) Gracilaria spp.) | 32–39 | 83–89 | Optimized Fe, Cu, Mg, Ca concn for microbial |
| Noble agar | Kanten, agar-agar | Agarose (70%) plus agaropectin (30%) | Red algae (<i>Gelidium</i> spp., Gracilaria spp.) | 32–39 | 83–89 | growth Washed and bleached agar |
| Carrageenan | Vegetable gelatin | 1,3α-1,4β-Galactans with one (κ-), two (ι-), or three (λ-) sulfates per disaccharide unit | Red algae (Kappaphycus alvarezii) | 3050 | 50-70 | Requires vigorous mixing to avoid clumping |
| Phytagel | Gellan gum, Nanogel-TC, Gelrich, Grovgel, AppliedGel, Gelrite | Bacterial substrate composed of glucuronic acid, rhamnose, and glucose | Bacterium (<i>Pseudomonas</i> elodea) | 3550 | >70 | Requires presence of Ca ²⁺ or Mg ²⁺ |
| Alginic acid | Algin, alginate | Linear copolymer with blocks of (1-4)-linked β -D-mannuronate and α -L-guluronate residues, linked together in different sequences or blocks | Brown algae | NA | NA | Did not gel |
| Guar gum | Guaran | Linear chain of β-1,4-linked mannose residues to which galactose residues are 1,6-linked at every second mannose | Guar or cluster bean (<i>Cyamopsis</i> tetragonoloba) | NA | NA | Did not gel |
| Gelatin | | Hydrolyzed collagen extract | Cattle, chicken, pigs, or fish | 25–35 | 2535 | Too-low melting temp, amino acid source |
| ^a NA, not applicab | le. | | | | | |

15 min at 121°C before use; selective drugs were added after the liquid gel solution cooled to below 60°C in a water bath. Data for the range of typical element components of agarose, Bacto agar, Noble agar, and Phytagel were obtained from Difco (24) and for carrageenan (potassium, sodium, calcium only) from the Sigma-Aldrich website. Additional, lot-specific cation concentration information was obtained from the Certificates of Analysis from BD Biosciences and Sigma-Aldrich, respectively.

Colony imaging. White-light images of gels and yeast colonies were acquired using a digital imaging setup described previously (11) with a commercially available single-lens reflex (SLR) camera (18-Mpixel Rebel T3i; Canon USA Inc., Melville, NY) with an 18-to-55-mm zoom lens. We used a white diffuser box with bilateral illumination and an overhead mount for the camera in a darkroom. Colony information was collected after images were normalized, spatially corrected, and quantified using a set of previously published custom algorithms, also known as the Colony Analyzer Toolkit (11). Digital images were cropped and assembled in Photoshop and Illustrator (CS5; Adobe Inc., San Jose, CA) for publication. Fluorescent images of gels were acquired using a custom fluorescent digital imaging setup. We used a commercially available SLR camera (20.2-Mpixel EOS 6D; Canon) with a 100-mm f/2.8 macro lens (Canon) and a green band-pass filter (BP532; Midwest Optical Systems, Inc., Palatine, IL). We used a 460-nm LED panel (GreenEnergyStar, Vancouver, BC, Canada) with a 1/4 white diffusion filter (251; Lee Filters, Burbank, CA, USA) for 45° bilateral illumination (205560; Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany) and an overhead mount for the camera (205510; Kaiser) in a darkroom.

Spectral absorbance measurements. For spectral absorbance measurements, 200 μ l of gel was prepared as described above and poured into 96-well, UV-transparent microtiter plates (3635; Corning Inc., Tewksbury, MA) and allowed to solidify. Absorbance measurements were taken in triplicate in sweeps from 350 nm to 750 nm on a SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

Bioavailability screen in *S. cerevisiae* **S288C/BY4741.** To test how available nutrients are in the different media, gels were prepared as described above and poured into sterile rectangular high-throughput screening plates (PlusPlates V2; Singer Instrument Co. Ltd., Watchet/ Somerset, United Kingdom). Randomly selected yeast deletion mutants (n = 1,536) from the Yeast Knockout Deletion Collection (46) (YKO; GE Dharmacon, Lafayette, CO, USA) were grown on SC supplemented with G418 (100 µg/ml), and the plates were then pinned using the Rotor HDA (Singer) onto the various gel substrates (without any selection markers). Plates were grown at 30°C for 24 h, and colony sizes were measured using the white-light colony imaging station described above.

Bioavailability screen in diploid *S. cerevisiae* **and** *Saccharomyces* **paradoxus wild-type strains.** To test if nonstandard laboratory or wild yeast strains are able to digest the gel material and utilize it as a carbon or a nitrogen source, we prepared gels as described above and poured them in sterile 10-cm petri dishes. We then streaked out 12 yeast strains (6 *S. cerevisiae* and 6 *S. paradoxus* strains; see Fig. S2 in the supplemental material) onto each gel and recorded the qualitative growth and appearance of the colonies after 72 h at 30°C. The strains were a gift from Scott Rifkin and were initially acquired from the National Collection of Yeast Cultures (Norwich, United Kingdom).

Gel strength measurements. Gel strength measurements were performed using a TA.XT2.plus texture analyzer (Stable Micro Systems, Godalming/Surrey, United Kingdom). Gels were prepared as described above in 100 ml of ddH₂O in a standard 100-ml Pyrex Griffin glass beaker. After pouring, the gels matured and solidified for 24 h at 4°C. Prior to the gel strength measurements, gels were allowed to warm to room temperature. The following settings were used for the TA.XT2.plus analyzer: pretest speed, 5 mm/s; test speed, 1 mm/s; posttest speed, 5 mm/s; target mode, distance; distance, 2 mm; time, 5 s; trigger type, force; and trigger force, 5 g (weight). We used a standard 12.7-mm-diameter, flat, sharp-edged plunger (TA-10; Texture Technologies, Hamilton, MA) in the center of the gel. The gel strength reported is the weight (in grams) required to depress the gel by 2 mm. Measurements were recorded with Exponent (Stable Micro Systems). Contact angle α was defined as the angle formed between two lines drawn on the colony cross section. The first line was drawn from the point where the colony sides touch the gel surface on the left side of the colony to the point where the colony sides touch the gel surface on the right side of the colony; the second line was drawn from the point where the colony sides touch the gel surface on the left side of the colony outline where the colony reaches its half-maximum colony height.

Gel pH measurements. Gel pH measurements were performed in triplicate with a double-junction, flat-bulb, gel-filled epoxy electrode (A57184; Beckman Coulter, Brea, CA) in full contact with the gel surface. Gelling, supplemental, and medium reagents were prepared as described above. A 10-ml sample of each gel was added to 20-ml glass scintillation vials (Wheaton, Millville/NJ), sealed, and allowed to harden overnight at 4°C. Measurements of pH were performed on a PHI510 benchtop pH meter (Beckman Coulter) after the gels returned to room temperature and were allowed to equilibrate in ambient air for ~1 h.

Diffusion measurements. Using the fluorescent gel imaging station, diffusion was assessed by tracking the spread of fluorescein sodium salt (fluorescein isothiocyanate [FITC]) (F6377; Sigma-Aldrich) in the gels. Gelling reagents were prepared as described above and poured into sterile 10-cm petri dishes. The gels were allowed to harden overnight at 4°C. The next day, the gels were warmed to room temperature and three circular holes were punched into the center of each gel. A total of 110 μ l of fluorescein sodium salt solution (2 mM) was then pipetted into the holes and allowed to diffuse into the gel. Images were acquired every 5 min for a period of 12 h.

Drug availability screen. To test the availability of common selection markers in the gel substrates, gels were prepared as described above [using SC without lysine or arginine but with monosodium glutamate (SC-lys-arg+MSG) and without (NH₄)₂SO₄] and poured into sterile rectangular high-throughput screening plates (PlusPlates V2; Singer) supplemented with increasing concentrations of G418, clonNAT, S-AEC, or Can (0, 10, 20, 40, 100 µg/ml [endpoint for S-AEC and Can], 200, 400, and 1,000 µg/ml). The test yeast strains with the following genotypes (based on the *S. cerevisiae* strain BY4742/S288C) were grown to saturation in yeast extract-peptone-dextrose (YPD) medium overnight: strains A (XXX = *his*3 Δ) and B (XXX = *ho* Δ), *MATa his*3 Δ 1 *leu*2 Δ 0 *ura*3 Δ 0 LYS2⁺ *can*1 Δ ::STE2pr-SpHIS5 *lyp*1 Δ ::STE3pr-LEU2 XXX::NatMX; strains C (YYY = *his*3 Δ) and D (YYY = *ho* Δ), *MATa his*3 Δ 1 *leu*2 Δ 0 *ura*3 Δ 0 *met*15 Δ 0 LYS2⁺ CAN1⁺ LYP1⁺ YYY::KanMX.

Twelve serial 2-fold dilutions (1:1 to 1:2¹¹) of the overnight liquid culture were pinned onto the respective gels, and colony growth was quantified after 24 h at 30°C by counting the total number of colonies with visible growth (out of 12). For example, if 12 G418-resistant colonies had grown up at a given concentration of clonNAT compared to 12 clonNAT-resistant colonies, then that would equate to 100% viability relative to the resistant strain (12/12). If at another concentration of clonNAT, 4 G418-resistant colonies had grown up compared to 12 clonNAT-resistant colonies, then that would equate to 33% viability relative to the resistant strain (4/12).

High-throughput yeast screens. To assess the colony sizes of a large selection of yeast mutants with different fitness on the gel substrates, we pinned a random selection of 1,536 strains from the Yeast Knockout Deletion Collection (46) (YKO, Dharmacon) onto standard Bacto agar plates with 100 µg/ml G418 [SC+MSG without (NH₄)₂SO₄]. From these plates, colonies were then transferred onto the indicated gel substrates [100 µg/ml G418 in SC+MSG without (NH₄)₂SO₄]. Colony sizes were correlated between Bacto agar and all other substrates. Interplate correlations were calculated by computing Pearson's correlation between samestrain colony sizes (n = 1,536 per plate). A detailed description of the colony array procedure for the 1,536 strains (see Fig. S1 in the supplemental material) is as follows. The 1,536 yeast strains were stored as liquid glycerol stocks in 16 96-well microtiter plates. After thawing, yeast aliquots were transferred onto rectangular Bacto agar (with YPD) gels with a standard microtiter footprint (12.7 cm by 8.5 cm) by use of a disposable



FIG 1 Gel appearance and optical qualities. (A) Gels were prepared either with no components added (plain) or with common medium components added (Glu, glucose; SC, synthetic complete mix; YP, yeast extract and peptone) (scale bar, 10 mm). An empty set of vials was added at the bottom to create comparable lighting conditions for all gels (only lids are visible at the bottom). (B and C) To assess the optical quality of gels with growth media, i.e., SC (C) and YP (D), absorbance was measured from 350 nm to 750 nm. (D) Side views of typical high-throughput yeast screening plates prepared with the different gels demonstrate the range in gel clarity (same concentrations as in panel A; scale bar, 4 mm). Yeast colonies are hanging from the gel slab; differences in the optical path make colonies appear twice (red arrows in the schematic).

plastic pinning tool ("pads") with 96 individual long pins in a pneumatically operated pinning robot (Rotor HDA; Singer). The yeast colonies were grown as individual microcolonies (~0.2 to 3 mm in diameter). During successive rounds of overnight growth at 30°C and repinning, the colonies were then combined into increasingly higher colony densities by using 96-pin and then 384-pin pads. Colonies were then replicated onto the various gel substrates using 1,536-pin pads. This process is extremely fast (1,536 colonies are replicate-pinned in under 30 s) and highly scalable (i.e., colonies can be arrayed in higher or lower colony densities and transferred between conditions).

Autofluorescence measurements. Autofluorescence measurements were taken using the fluorescent imaging station described above, using an average pixel intensity of a 20- by 20-pixel area in the center of the jar as the readout. Images of green fluorescent protein (GFP)-expressing yeast colonies were all taken at identical camera settings, and signal-to-noise ratios were calculated based on the ratio between the low- or high-GFP signals after background subtraction. The low- or high-GFP-expressing strains were a gift from Randy Hampton (UCSD) and based on the *S. cerevisiae* strain BY4742/S288C. Basal GFP fluorescence was produced by

a strain with an *ade2-101 met2 lys2-801 trp1::hisG leu2* $his3\Delta 200$ *ura3-52::URA3::HMG2*-GFP genotype; increased GFP fluorescence was produced by a strain with an *ade2-101 met2 lys2-801 trp1::hisG leu2* $his3\Delta 200$ *ura3-52::URA3::HMG2*-GFP *hrd1* Δ ::KanMX genotype.

RESULTS

After following previously published protocols (39, 41), we were unable to obtain properly solidified gels when using alginic acid or guar gum, despite testing a wide range of substrate concentrations and mixing methods (2 to 10%; data not shown). Similarly, even high gelatin concentrations (16%) yielded only semisolid gels (47), and gelatin contains large amounts of arginine, lysine, and histidine (48) that can be freed through yeast proteinase digestion (49), rendering it unusable for auxotrophic selection. Consequently, alginic acid, guar gum, and gelatin do not provide an acceptable gel quality for high-throughput screening and were thus excluded from further analysis.

Gelling agents vary widely in their optical qualities. All agar-



FIG 2 Nutrient availability and colony characteristics. (A) Yeast colonies were grown for 24 h on gels with different levels of nutrients to assess if the gels release anabolites. Gels were prepared without any additional nutrients (gel only), with a carbon source (+Glu), with a nitrogen source (+YP), or with full nutrient media (+Glu +SC and +Glu +YP). Orange crosshairs indicate the colonies analyzed in panel B. The same nine colonies are shown under all conditions (scale bar, 4 mm). (B) Cross sections of colonies in panel B. α , contact angle of the colony edge with the surface. (C) Mean raw colony sizes \pm standard error of the mean (SEM) of 1,536 mutant yeast strains grown on the different gels (+Glu +YP) for 24 h. All mean colony sizes are significantly different from each other at a *P* of <0.001 except those grown on Bacto agar and Noble agar (one-way analysis of variance [ANOVA], followed by Tukey's comparison of all pairs).

based gels (agarose, Bacto agar, Noble agar) result in gels with a hazy quality, while carrageenan and Phytagel gels are much clearer (Fig. 1A). We quantified the absorbance of the gels from near UV (350 nm) to near infrared (750 nm) and found a peak absorbance in the UV range for these types of gels (Fig. 1B and C). Agar-based gels exhibit dramatically stronger absorption and scattering than both carrageenan and Phytagel gels, which can be easily appreciated when a gel plate with yeast colonies is examined from the side (Fig. 1D).

Different gels are not a significant source of nutrients. One major concern when evaluating new gelling agents is to unknowingly introduce nutrients into the experiment. To test whether any of the gels are able to provide nonsupplied nutrients, we tested the growth characteristics of 1,536 yeast strains from the yeast deletion collection for their ability to grow on plain gel, gel supplemented with only a carbon source (glucose), or gel supplemented with only a nitrogen and vitamin source (yeast extract and peptone). We found no growth under nutrient-limited conditions for any gel, indicating that they are not a significant source of nutrients (Fig. 2A). To ensure that this observation was not an artifact of the highly auxotrophic laboratory strains used in the yeast gene deletion collection, we also tested the growth of 12 diploid wildtype S. cerevisiae and S. paradoxus strains and observed similar results (see Fig. S2 in the supplemental material). To what extent these results hold true for other, nonyeast microbes will have to be determined on an individual case-by-cases basis.

Colony morphology differs between gels. All gels tested allowed the growth of clearly identifiable, almost perfectly round yeast colonies. To identify potential changes in colony morphology and specifically colony width and height, we investigated colonies grown under nutrient-rich conditions more closely (Fig. 2A). Using intensity as a measure of colony thickness, we compared colony cross sections between the gels and found that agarbased colonies exhibit almost identical colony shapes, while carrageenan- and Phytagel-grown colonies are taller and have a steeper contact angle α between the edge of the colony and the gel surface (Fig. 2B). This more vertical distribution of biomass leads to overall smaller colonies that are consequently more widely spaced apart on carrageenan and Phytagel gels (Fig. 2C).

Gel pH and gel strength. To investigate the biophysical properties that might underlie the observed changes in colony morphology, we measured gel pH and gel strength of the different gels used. While the plain gels differ quite widely in their initial pH (\sim 5 to 6.5), the addition of buffering media, such as yeast extract and peptone or SC, results in gels with a much more narrow pH range (6.1 to 6.3 and 4.2 to 5.2) (Fig. 3A). These findings suggest that pH alone does not differentiate the gel types.

Next, we measured gel strength (the weight [in grams] necessary to depress the gel surface by 2 mm) and found it to vary greatly between gels (\sim 200 to \sim 1,800 g) (Fig. 3B). Gel strength has previously been reported to influence the contact angle α between yeast colonies and the gel surface (50), which could explain the much steeper and higher colonies on Phytagel; however, it does not explain the behavior of the carrageenan-grown colonies.

Gel diffusion is dependent on gel concentration, not gel strength. Nutrients, waste products, and drugs need to diffuse



FIG 3 Gel pH and strength. (A) Gel pH was measured under gel-only conditions or with different medium components added (Glu, glucose; SC, synthetic complete mix; YP, yeast extract and peptone). (B) Gel strength was measured in plain gels as the weight (in grams) necessary to depress the gel by 2 mm.

through the gel matrix to enable colony growth. To assess if differences in diffusion exist between the gel types, we performed a radial diffusion experiment using fluorescein isothiocyanate $(M_w = 389.38 \text{ g/mol})$ as a molecular tracer (Fig. 4A). We measured the diameter of the diffusion disc at given intervals and observed that the diffusion kinetic is well described in terms of the Higuchi equation, which models the diffusion of drug into gel as a perfect sink (51) (Fig. 4B). The speed of diffusion was proportional to the concentration of gelling substance used, but it was independent of gel strength or substrate (Fig. 4C).

Drug bioavailability is affected by gel substrate. Microbial experiments frequently rely on selective drugs to maintain certain cellular populations. However, some differences of drug bioavailability in different gel media have been reported (52). To assess if commonly used drugs are equally effective in the different gelling agents tested, we prepared plates with increasing concentrations of nourseothricin (clonNAT), Geneticin (G418), *S*-(2-amino-ethyl)-L-cysteine hydrochloride (S-AEC), and canavanine (Can). We then prepared dilution series of liquid overnight cultures of strains sensitive or resistant to the tested drugs and plated those

onto the gels. Colony growth was evaluated relative to the respective drug-resistant strain (Fig. 5A). We found no difference between $his3\Delta$ and $ho\Delta$ strains (data not shown) and therefore subsequently combined these measurements.

Can and S-AEC (toxic amino acid analogues) are largely unaffected by the gel substrate in their ability to severely impair yeast growth (Fig. 5B and C). Surprisingly, we observed a dramatic difference between gel types when we evaluated the toxicity of the aminoglycoside antibiotics G418 and clonNAT. A commonly used concentration for these antibiotics is 100 μ g/ml. We found that agarose and Noble agar facilitate drug toxicity and kill sensitive yeasts at antibiotic concentrations as low as 10 µg/ml to 40 µg/ml (Fig. 5D and E). In contrast, Bacto agar, carrageenan, and Phytagel failed to completely kill sensitive yeasts in a range up to 100 μ g/ml (Fig. 5D and E), prompting us to extend the tested antibiotic concentration range and to compare SC and YPD-based media (Fig. 5F and G). With YPD as a medium base, G418 on Bacto agar does indeed exhibit complete toxicity at the standard 100 µg/ml; however, SC requires 200 µg/ml for complete toxicity (Fig. 5F), even in the absence of ammonium sulfate as used here.



FIG 4 Diffusion characteristics. (A) A small fluorescent tracer molecule was allowed to diffuse into the gel matrices for 12 h (scale bar, 10 mm). (B) Diffusion diameter was measured at the indicated time points. In agreement with the Higuchi equation, diffusion is proportionate to the square root of time and appears to be determined only by gel concentration (%, wt/vol). (C) Relationship between gel strength and the slope of the diffusion fit.



FIG 5 Drug bioavailability. (A) Example of a drug bioavailability experiment. Strains susceptible ($his3\Delta$::KanMX) or resistant ($his3\Delta$::cNAT) to clonNAT were diluted (1:1 to 1:2¹¹) and spotted onto increasing concentrations of clonNAT-containing Bacto agar with SC plates (without ammonium sulfate). Images are examples of underlying data in panel E. (B) Titration curve for canavanine on SC plates. Viability is measured relative to the number of colonies from a drug-resistant strain at the same drug concentration. (C) Titration curve for S-AEC on SC plates. (D) Titration curve for G418 on SC. (E) Titration curve for G418 in YPD. (G) Titration curve for clonNAT on YPD. (H) Estimated element content of typical batches of the gel substrates based on supplier specifications. No data are available for carrageenan magnesium content. A red "X" indicates lot-specific cation content based on Certificate of Analysis (CoA) where available. (I) Relationship between the clonNAT dosage that kills 6/12 dilution colonies (y axis) and the total cation content of the underlying gel substrate (x axis). Results are shown for SC media, except carrageenan, for which the YPD data point was used.

Similarly, G418 on Phytagel exhibits toxicity at 400 μ g/ml with YPD and at 1,000 μ g/ml with SC (Fig. 5F). Remarkably, we were unable to induce any toxicity in the test range using G418 on carrageenan plates (Fig. 5F). Using clonNAT as the antibiotic resulted in similar findings (Fig. 5G).

Typically, G418 is used in media lacking ammonium sulfate, which has been reported to impair G418 toxicity (53). We thus speculated that cation content of the gel could play a role in the bioavailability of particular drugs. This observation is in agreement with earlier reports of cation interference with aminoglycoside antibiotics in Pseudomonas aeruginosa (54, 55); however, gelling substrate and nutrient mixes as a potent cation source had so far not been appreciated. While there is no evidence of significant ammonium content in the gel substrates, the gels do vary by multiple orders of magnitude in their potassium, sodium, calcium, and magnesium contents (Fig. 5H), and the effective antibiotic dose is directly related to the overall cation concentration of the final gel (Fig. 5I). These findings suggest that natural mineral content or contaminations from the manufacturing process of the gel substrates can have a marked influence on the effect of some drugs (i.e., aminoglycoside antibiotics) but not others (i.e., amino acid analogues).

High-throughput array performance. To assess the suitability of the different gels as substrates for microbial high-throughput screens, we pinned 1,536 random mutant yeast strains from the yeast deletion collection onto standard Bacto agar plates and compared the normalized colony sizes ("fitness") between gel substrates. Six replicates of the same 1,536 colonies on Bacto agar yielded interplate correlations of 0.88 to 0.95 (Pearson's correlation) (Fig. 6A). To account for the gel strength differences observed in the gels (Fig. 3B), we adjusted gel concentrations slightly to achieve similar gel strengths in the test gels. Additionally, we tested two Phytagel mixtures, 1% Phytagel with 0.05% MgSO4 and 0.5% Phytagel with 0.1% MgSO₄. Intergel correlations were between 0.85 and 0.96 (Pearson's correlation) (Fig. 6B). Overall, the mutant fitness between gel substrates correlated very well. In the non-agar-based plates, we observed a slight trend to more centralized, less extreme fitness values, as indicated by the off-diagonal least-square fits (Fig. 6C) and tighter frequency distributions (Fig. 6D). These results suggest that there are no dramatic relative growth differences between the evaluated gel substrates.

Fluorescent high-throughput applications. Numerous fluorescent marker and reporter systems have been developed in bacteria and yeast (56–58), and it would be highly desirable to utilize them in a high-throughput screening setting. To evaluate the fluorescent properties of the gels, we imaged the gel jars with various medium supplements by use of a fluorescent imaging setup, revealing substantial differences in autofluorescent properties (Fig. 7A). While the plain gels themselves have little autofluorescence, once supplemented with medium, the agar-based gels are about twice as autofluorescent as the carrageenan or Phytagel gels (Fig. 7B). This increased fluorescence is likely due to enhanced scattering in the more opaque agar-based gels. Additionally, in SC-based gels, the effect may be due to the absence of quenching compounds in the less defined, extract-based YP media (Fig. 7B). To assess the effects of gel autofluorescence on substrate usability for fluorescent screens, we plated low- and high-GFP-expressing yeast colonies on the different gels supplemented with yeast extract, peptone, and glucose (Fig. 7C). While gel autofluorescence was not additive to high or low colony signals, the background



FIG 6 High-throughput array performance. (A) A random selection of 1,536 mutant yeast strains from the deletion collection was grown in parallel on six Bacto agar 2% plates. Correlation between colony sizes on the six "identical" plates ranged from 0.88 to 0.95 (Pearson correlation). The mean colony size from these six plates was set as the "gold standard" for panels C and D. (B) Each of the six plates was pinned onto a test plate with the indicated gel substrate, and colony sizes were correlated. Gel concentrations were adjusted slightly to provide similar gel hardness. (C) Scatter plots between the fitness of the indicated gels (spatially corrected and plate mode-normalized colony sizes) and the Bacto agar "gold standard" (*r*, Pearson correlation). A gray line marks the diagonal, and the orange line indicates the least-squares fit of the scatter data; colonies smaller than 0.05 mm were excluded. (D) Histograms of the fitness values for each gel substrate compared to the "gold standard."

intensities differed widely between substrates. Thus, it is possible for this background fluorescence to wash out very low GFP signals, such that reduced autofluorescence may make colony edge detection, size determination, and ultimately effect quantification much easier (Fig. 7D).



FIG 7 Autofluorescence properties. (A) Autofluorescence of the gels varies widely. Gels were prepared either with no components added (plain) or with common medium components added (Glu, glucose; SC, synthetic complete mix; YP, yeast extract and peptone) (scale bar, 15 mm). An empty set of vials was added at the bottom to create comparable lighting conditions for all gels (only lids are visible at the bottom). (B) Autofluorescence intensity of the gels relative to that of Bacto agar +SC + Glu (a common screening medium). (C) Examples of low- and high-GFP-expressing yeast strains on the indicated gel substrates (+YP + Glu) (scale bar, 3 mm). (D) Intensity cross sections (green channel) on the various media to exemplify signal-to-noise ratio of the different gel substrates from panel C.

DISCUSSION

After more than 100 years of heavy agar use in microbial research, we have taken a second look at agar's usefulness for today's more advanced high-throughput techniques. Certainly, Bacto agar is an established and versatile growth substrate that rightfully enjoys widespread acceptance. However, given the specialized needs of modern genetics screening experiments, we have found that other gel substrates provide highly beneficial properties.

In particular, Phytagel and carrageenan exhibit optical clarity that is vastly superior to that of agar-based gels, allowing for scatter-free through-gel image acquisition and improved fluorescent imaging. Additionally, Phytagel and carrageenan lead to smaller, better-defined colonies that enable higher colony densities, which may, in turn, lessen the risk of colony fusions. This is a highly beneficial property when pursuing super-high-colony densities. Instead of depending just on gel strength (50), a combination of biophysical properties (gel strength and/or cation content) appears to determine colony morphology: harder Phytagel and softer carrageenan have better-defined colonies and both are cation-rich. Agarose and Noble agar, on the other hand, are particularly low in salt contaminants, which appears to increase drug activity for certain drug types. This effect is likely mediated through binding of cations to the yeast cell wall, as receptor-mediated drug uptake is unaffected; however, the precise mode of action should be investigated further. Whenever drug cost or solubility is an issue, the use of these low-cation substrates should strongly be considered. For example, instead of the standard 100- μ g/ml concentration of clonNAT required for selection on Bacto agar SC plates, one could perform the same selection on agarose with 10 μ g/ml. Finally, additional savings can be realized since many substrates are less expensive than Bacto agar. In terms of biological gel-gene interactions, we find no evidence that switching from Bacto agar to any of the other substrates introduces any significant bias in the observed fitness of yeast mutants, further encouraging the use of these gel alternatives.

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P.A.J. designed the experiments. P.A.J., C.M., and L.R.W. performed the experiments. P.A.J. and T.I. wrote the article.

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