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Identification of novel antimicrobials using a live-animal infection model

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The alarming increase of antibiotic-resistant bacterial pathogens points to the need for novel therapeutic approaches to combat infection. To discover novel antimicrobials, we devised a screen to identify compounds that promoted the survival of the model laboratory nematode *Caenorhabditis elegans* infected with the human opportunistic pathogen *Enterococcus faecalis*. *E. faecalis* colonizes the nematode intestinal tract, forming a persistent lethal infection. Infected nematodes were rescued by antibiotic treatment in a dose-dependent manner, and antibiotic treatment markedly reduced the number of bacteria colonizing the nematode intestine. To facilitate high throughput screening of compound libraries, we adapted a previously developed agar-based *C. elegans*-*E. faecalis* infection assay so that it could be carried out in liquid medium in standard 96-well microtiter plates. We used this simple infection system to screen 6,000 synthetic compounds and 1,136 natural product extracts. We identified 16 compounds and 9 extracts that promoted nematode survival. Some of the compounds and extracts inhibited *E. faecalis* growth *in vitro*, but, in contrast to traditional antibiotics, the *in vivo* effective dose of many of these compounds was significantly lower than the minimum inhibitory concentration needed to prevent the growth of *E. faecalis* *in vitro*. Moreover, many of the compounds and extracts had little or no effect on *in vitro* bacterial growth. Our findings indicate that the whole-animal *C. elegans* screen identifies not only traditional antibiotics, but also compounds that target bacterial virulence or stimulate host defense.

antibiotic resistance | chemical genetics | *Enterococcus faecalis* | anti-infectives

The growing problem of antibiotic-resistant bacteria (1–4) and the imminent threat of biowarfare agents (5) point to a need for new anti-infective therapies. However, the rate of new antimicrobial discovery is unlikely to meet the expected need for the foreseeable future (6–10). Specific problems include the overmining of cultivable microorganisms (11), a high background of toxic compounds or compounds with poor pharmacokinetic properties in synthetic compound libraries (7, 12), the inability of most synthetic leads to penetrate across the multidrug resistance (MDR) barrier of Gram-negative bacteria (13, 14), and the use of *in vitro* assays for small-molecule discovery that bear little resemblance to the biological systems in which the drugs need to function (12, 15, 16). Reasoning that some of these obstacles might be overcome by screening directly in a live-animal infection model, we developed an assay for identifying compounds that promote the survival of the nematode *Caenorhabditis elegans* persistently infected with the human opportunistic bacterial pathogen *Enterococcus faecalis*, an infection that leads to nematode death.

C. elegans is a useful and simple model host that can be infected and killed by a remarkably large number of human pathogens, including the Gram-negative bacteria *Pseudomonas aeruginosa* and *Salmonella enterica*, the Gram-positive bacteria *E. faecalis* and *Staphylococcus aureus*, and the fungal pathogen *Cryptococcus neoformans* (17, 18). Each of these organisms has been studied in the *C. elegans* model by simply replacing the normal food source (the lawn of *Escherichia coli*) with the pathogen in question and mon-

itoring the survival of the nematodes. This simple feeding-based pathogenicity assay facilitates high-throughput screening and genetic analysis. Previous work has shown that there is broad overlap between the bacterial virulence factors required for pathogenesis in mammals and for *C. elegans* killing (18, 19). Additionally, the *C. elegans* pathogenesis models demonstrate that key features of the innate immune response have been conserved between *C. elegans* and mammals (20, 21).

Here, we show that nematodes infected with the persistent colonizer *E. faecalis* die when transferred to liquid medium in 96-well plates but can be rescued by treatment with antibiotics. We used this infection system to identify synthetic compounds and natural product extracts that promoted host survival. Advantages of this model are the ability to discover compounds with no antimicrobial activity *in vitro*, including prodrugs or compounds that target functions important only for *in vivo* survival or virulence, or activators of innate immunity. Because the readout of this *in situ* assay is nematode survival, an additional advantage of the screen is that it decreases the large background of compounds that are toxic or ineffective *in vivo* due to poor pharmacokinetics. Thus, this assay not only identifies compounds with novel antimicrobial activities but also has the potential to solve the bottleneck of toxicity/efficacy testing in drug development.

Results

Antibiotic Treatment Rescues Infected Nematodes. When *C. elegans* are transferred from a lawn of *E. coli*, their normal laboratory food, to a lawn of *E. faecalis* growing on agar medium, the *E. faecalis* forms a persistent and lethal infection in the *C. elegans* intestine (22), surviving digestion and accumulating to levels up to 10⁵ colony-forming units (cfu) per worm. The infection persists in the worms even after they are transferred to a benign bacterial food source such as *Enterococcus faecium* that does not kill worms (22). We established an agar-based persistent infection model to determine whether antibiotic treatment could cure worms of an *E. faecalis* infection. In these experiments, *Enterococcus faecium*, which accumulates in the *C. elegans* intestine, but which does not kill the worms or persistently colonize (22), was used as a negative control. Worms were fed on lawns of *E. faecalis* or *E. faecium* and transferred to fresh lawns of *E. faecalis* or *E. faecium*. The rationale for transferring the worms from *E. faecalis* to *E. faecium* was that worms could feed on the nonpathogenic *E. faecium* strain but would remain colonized with *E. faecalis*.

Worms transferred from *E. faecalis* to *E. faecalis* died with similar kinetics as worms left on an *E. faecalis* lawn for the entire duration of the experiment; half of the worms died within 5 days (Fig. 1*A* and data not shown). Worms transferred from *E. faecalis* to *E. faecium* died because of the persistent infection, although the killing rate

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Abbreviations: cfu, colony-forming unit; MIC, minimum inhibitory concentration; BHI, brain–heart infusion; MAPK, mitogen-activated protein kinase; LT₅₀, time for half to die.

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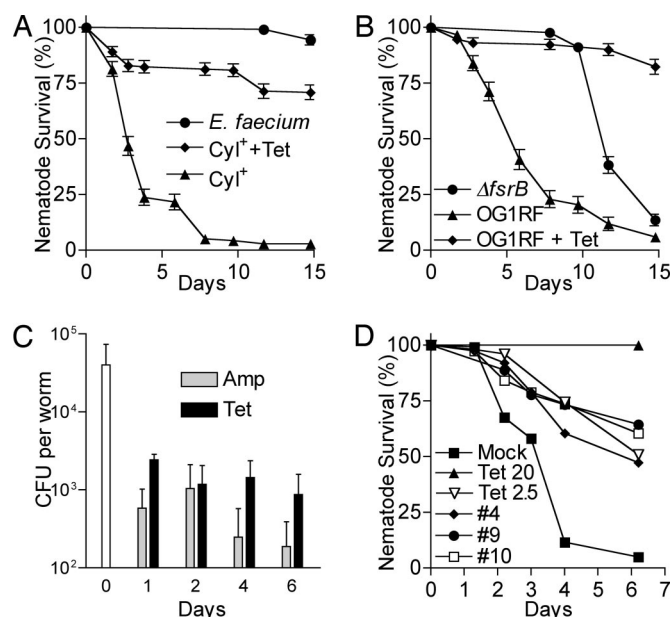


Fig. 2. The liquid infection assay gauges differences in nematode killing due to *E. faecalis* strains with varying degrees of pathogenicity or due to antibiotic treatment. (A) Kaplan–Meier survival curves of *glp-4;sek-1* nematodes that were infected with the cytotoxin (Cyl) producing *E. faecalis* strain MMH594 (▲, ◆) or the *E. faecium* strain 11M12 (●). Cyl-infected nematodes were treated with 0 (▲) or 20 μ g/ml (◆) tetracycline (Tet). Error bars equal SEM. (B) Kaplan–Meier survival curves of *glp-4;sek-1* nematodes that were infected with *E. faecalis* OG1RF (▲, ◆) or the two-component quorum-sensing regulator mutant OG1RF Δ *fsrB* (●). OG1RF-infected nematodes were treated with 0 (▲) or 20 μ g/ml (◆) tetracycline. (C) The bacterial load in the nematode intestinal tract after antibiotic treatment. *E. faecalis*-infected nematodes were treated in liquid media containing 20 μ g/ml ampicillin (Amp) or tetracycline, and the number of cfu per worm was determined. Error bars equal standard deviations. (D) Curing kinetics of selected hit compounds. Shown are Kaplan–Meier survival curves of infected nematodes treated with 25 μ g/ml compound 4 (◆), 50 μ g/ml compound 9 (●), 25 μ g/ml compound 10 (□), 2.5 μ g/ml tetracycline (▽), 20 μ g/ml tetracycline (▲), or mock treatment (■). In pairwise comparisons to mock treatment using log-rank tests, the difference for all of the treatments was significant, with $P < 0.0001$.

kill nematodes in the liquid assay, and *E. faecalis* strains that produce cytotoxin killed more quickly in the liquid assay. The strain that showed the fastest killing was the cytotoxin-positive strain MMH594 (27). The LT_{50} of *glp-4;sek-1* worms infected with MMH594 was 2.8 days (Fig. 2A), and this strain was used in the compound screening assays described below. Importantly, worms infected with the three different *E. faecalis* strains could be rescued with tetracycline, as illustrated in Fig. 2A and B.

We also examined the effect of antibiotic treatment on the colonization of *E. faecalis* in the nematode intestinal tract. After feeding on *E. faecalis* OG1RF for 16 h, an average of 4.0×10^4 cfu was recovered from each worm (Fig. 2C). The worms were then transferred to liquid media containing 20 μ g/ml ampicillin or tetracycline or no antibiotic. Although the number of viable bacteria that colonized the untreated worms remained relatively constant for 2 days of incubation in liquid media (data not shown), the number of cfu per worm in ampicillin or tetracycline-treated worms declined dramatically. After 24 h of treatment, the cfu per worm in ampicillin- and tetracycline-treated worms were only 1.5% and 6.1% of the cfu in untreated worms, respectively. After 6 days of treatment, the percentage dropped to 0.6% and 3.6% for the ampicillin- and tetracycline-treated worms, respectively (Fig. 2C). Interestingly, the small numbers of worms that appeared sickly or that died with antibiotic treatment were colonized with the same numbers of *E. faecalis* as untreated worms.



Fig. 3. Scoring live/dead worms in the liquid killing assay. Living nematodes in the liquid infection assay maintain a sinusoidal shape, whereas dead nematodes in the liquid infection assay appear as straight, rigid rods as the corpse becomes filled with bacteria.

In the liquid assay, live worms maintain a sinusoidal posture (Fig. 3) and can be seen moving their body and pumping their pharyngeal muscles. In contrast, dead worms become straight and rigid as they become bloated with *E. faecalis* cells. Frequently, the dead and dying worms become so bloated that the body of the worms is stretched and lengthened or the vulva of the worm bursts out (Fig. 3). These obvious differences in appearance between live and dead worms greatly facilitated the scoring of the assay.

Screening for Antiinfectives That Promote Survival of *E. faecalis*-Infected Worms. We performed a pilot screen in a 96-well plate format using the *E. faecalis*–*C. elegans* liquid infection assay for antiinfective substances that permitted the survival of the infected worms. We screened 6,000 individually synthesized compounds obtained from ChemBridge (San Diego), representing a rich variety of structures, and 1,136 natural product extracts from a National Cancer Institute (NCI) library that primarily contains extracts from tropical plants as well as extracts from marine invertebrates and algae. We manually examined the assay plates using a dissecting microscope after 6–8 days of incubation when 80–85% of the untreated worms have died. The false-positive rate of the screen was 2.7%. The false-negative rate using antibiotic controls or worms infected with the attenuated Δ *fsrB* strain was 1.7%.

The screen of the 6,000 ChemBridge compounds and 1,136 NCI extracts was carried out in duplicate, and a compound was scored as a “hit” if $>50\%$ of the worms survived in both duplicates or if one of the duplicate wells showed a high level of rescue (approximately $>80\%$ rescue). The 50% cutoff for this primary screen was not stringent, to ensure that substances with at least some activity would not be excluded. The primary screen identified 90 small molecule compounds (1.5% hit rate) and 58 extracts (5.8% hit rate). In a secondary screen, 18 of the small molecule compounds (0.3%) and 9 of the extracts (0.9%) were reconfirmed to promote survival of infected worms. For further testing, additional samples were purchased from ChemBridge. The structures, activity in the worm infection assay, and MIC values of 16 of these hits are shown in Table 1 and Fig. 4.

Among the 16 ChemBridge compounds, 15 did not exhibit any obvious toxicity toward *C. elegans*, at least with respect to overall appearance and health of the worms. The only compound displaying toxicity (compound 6) resulted in some growth retardation. However, none of the 16 exhibited cytotoxicity against sheep erythrocytes when tested in a hemolysis assay (data not shown). Importantly, 13 of the 16 compounds exhibited significant activity, increasing survival by at least 2-fold when retested in the curing assay (Table 1 and Fig. 4). Five of the compounds (compounds 1, 7, 8, 9, and 12) increased survival 3-fold or more, a curing rate that is significantly above the threshold sensitivity of the assay. Fig. 2D shows example curing rates with three selected ChemBridge compounds. Although none of the ChemBridge compounds cured as effectively as 20 μ g/ml tetracycline, these compounds were as effective as 2.5 μ g/ml tetracycline. Note that tetracycline is one of the best penetrating antibiotics and is often used against intracellular pathogens. In this regard, vancomycin, an efficacious anti-*E. faecalis* antibiotic, is probably a more realistic benchmark compar-

be prodrugs, that affect the virulence of the pathogen, that suppress pathogen survival, or that enhance the immune response of the host. Because some of the identified compounds and extracts have significant activity only *in vivo* in the whole-animal assay, these data provide proof-of-principle for using a whole-animal screen in a drug discovery program to identify novel antimicrobial compounds.

Compounds with a Lower Effective Dose *in Vivo* than *in Vitro*. One of the most interesting features of many of the identified compounds is their unusual ability to promote nematode survival at concentrations that were much lower than their MIC value *in vitro*. In contrast, the effective dose in the *C. elegans* curing assay of all of the known antibiotics that we tested was severalfold higher than the *in vitro* MIC. The effective dosage in the nematode model is similar to the therapeutic concentration of most antibiotics in human blood serum, which is typically 5- to 20-fold higher than the MIC (24). Possible explanations for the discovery of highly effective *in vivo* compounds with the nematode model include the possibilities that (i) the screen selects for compounds that are concentrated in the worm gut, (ii) the *C. elegans* immune system makes the bacteria more susceptible to inhibition, or (iii) the compounds weaken the integrity of the bacteria so that they are more readily digested. However, it seems likely that each of these explanations would also apply to traditional antibiotics. An alternative explanation is that the *C. elegans* curing assay specifically identifies compounds that target functions mainly important for *in vivo* survival or virulence, or activators of innate immunity, and that compounds with these activities may be more common than traditional antibiotics. Natural or synthetic compounds that block the virulence of pathogenic microbes ("virulence antiinfectives") are a largely unexplored class of antimicrobial agents. The successful targeting of a virulence product is demonstrated in a recent paper by Hung *et al.* (28), in which a high-throughput screen was used to identify compounds that inhibit the activity of the *Vibrio cholerae* transcriptional regulator ToxT, which is required for expression of cholera toxin.

Compounds that inhibit *E. faecalis* virulence could potentially target expression or function of cytolysin, serine protease, gelatinase, the quorum-sensing pathway, or colonization of the bacteria in the worm gut. We did not detect inhibition of proteolytic or cytolytic activity of the bacteria grown on milk agar or blood agar plates by any of the 10 compounds that we identified that had a high MIC value (data not shown). A possible mode of action for these compounds is the inhibition of bacterial colonization, but this activity is difficult to distinguish from antibiotic activity, which also reduces bacterial colonization.

Compounds that function as immune enhancers may activate the *C. elegans* immune pathway downstream of a conserved p38 MAPK cascade. A cascade consisting of the *C. elegans* PMK-1, SEK-1, and NSY-1 proteins, corresponding to the p38 MAPK, and upstream MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK), is required for the response to a variety of bacterial and fungal pathogens, and loss of any of these signaling components results in nematodes that have enhanced susceptibility to the pathogens (25). The *C. elegans*-*E. faecalis* curing assay used a *sek-1* mutant worm strain that dies more quickly when exposed to *E. faecalis*, and a potential activity of a hit would be an activator of the downstream p38 MAPK.

Toxicity Testing. Fifteen of 16 compounds identified in this study did not show any signs of toxicity against *C. elegans* or mammalian erythrocytes, indicating that the screen was able to select against toxic compounds. In a separate study using the nematode infection assay, we found that the indole derivative INF55, which is an effective inhibitor of multidrug resistance (MDR) pumps, is toxic to *C. elegans*, in agreement with the finding that this compound is also toxic to HeLa cells at similar concentrations (P. Markham, personal communication). These results indicate that the worm

infection model will be able to select against at least some compounds that exhibit toxicity.

In other studies, *C. elegans* has been used as an indicator of toxicity from heavy metals, environmental pollutants, organic solvents, and neurotoxins (29). Toxicity against nematodes has been quantified based on nematode survival, growth, reproduction, expression of stress response proteins, feeding behavior, and movement. The utility of *C. elegans* in toxicology testing greatly depends on how it correlates with toxicity in mammalian models. Williams and Dusenbery (30) determined that toxicity of heavy metals against *C. elegans* as measured by the LC₅₀ values correlates well with toxicity against mice or rats in rank order tests. Additionally, Cole *et al.* (31) reported a significant correlation from rank order toxicity tests from organophosphates between *C. elegans* and rodents.

Conclusions. The advent of genomics and combinatorial chemistry brought with them the promise of using defined targets to identify new antibiotics (32). Follow-up chemistry was then supposed to produce modifications enabling an inhibitor to effectively penetrate pathogen cells. This approach, however, has proven to be a formidable problem. Our results suggest that *C. elegans* whole-animal antimicrobial screening may be an effective new drug-discovery platform.

There are a number of advantages of the *C. elegans* whole-animal screening method:

1. Nematodes can be infected by a variety of different pathogens, making the model amenable to identification of both narrow- and broad-spectrum antibiotics.
2. Compounds are automatically tested for host toxicity because the assay requires that the compound does not adversely affect the normal physiology of the nematodes.
3. Compounds are automatically tested for *in vivo* efficacy.
4. The screen automatically identifies prodrugs that have to be modified by the host to form an active antimicrobial.
5. The screen identifies compounds that target previously unidentified virulence factors (or other functions important for *in vivo* survival) but may not interfere with the growth of the pathogen *in vitro*.
6. The screen identifies compounds that enhance host immunity.

The data shown in Table 1 provide proof-of-principle that the *C. elegans* antimicrobial screen will produce hits that are overlooked in a conventional *in vitro* screen and may yield better-quality hits that have direct *in vivo* activity.

Materials and Methods

Bacterial and Nematode Strains. WT Bristol N2 (33) and *glp-4(bn2ts);sek-1(km4)* (34, 35) *C. elegans* strains were maintained by using standard practices (36). *E. faecalis* strains MMH594 (27), OG1RF (37), OG1RF Δ *fsrB* (26), V583 (38), VS583 (23), and *E. faecium* strains DO (39) and 11M12 (23) were grown on BHI media (Difco/Becton Dickinson) at 37°C.

Nematode Killing and Rescue. N2 or *glp-4(bn2ts);sek-1(km4)* worms were synchronized by isolating eggs from gravid adults, hatching the eggs overnight in M9 buffer, and plating L₁-stage worms onto lawns of *E. coli* on nematode growth medium (NGM) agar media. Worms were grown to sterile, young adults by incubation at 25°C for 48–52 h, washed off the plates with M9 buffer, resuspended and washed in M9 buffer, deposited onto lawns of *E. faecalis* grown on BHI agar plates containing kanamycin at 80 μ g/ml to inhibit *E. coli* growth, incubated for 8–12 h at 25°C, and resuspended in M9 buffer. For assays using agar media, $\approx$ 35 infected worms were washed and then deposited onto 35-mm plates containing the appropriate antibiotics. Plates were incubated at 25°C and scored for worm survival at regular intervals. Worms were considered dead if they were unre-

sponsive to touch with a platinum wire pick. For the initial assays using liquid media, ≈ 80 infected worms were transferred to wells of a six-well plate containing 2 ml of media consisting of 10% BHI 80 $\mu\text{g}/\text{ml}$ kanamycin, 90% M9 buffer, and the appropriate antibiotics. The plates were incubated without agitation at 25°C and 80–85% relative humidity. To score for worm survival, the six-well plates were shaken by hand, and the worms were considered to be dead if they did not move or exhibit muscle tone.

Bacterial Colonization. Infected worms were washed three times with M9 buffer containing 1 mM sodium azide to inhibit expulsion of bacteria from the worm intestine. Approximately 10 worms were transferred to a 2-ml microcentrifuge tube, and the volume was brought to 250 μl . Fifty microliters of buffer was removed and plated to determine the number of external cfu. Approximately 400 mg of 1.0-mm silicon carbide particles (Catalog no. 11079110sc; Biospec Products, Bartlesville, OK) were added to each tube, the tubes were vortexed at maximum speed for one minute, which disrupts the worms but does not affect bacterial survival, and the resulting suspension was diluted and plated onto selective media to determine cfu.

Screening for Antiinfectives. Synchronized L₄ stage to young adult *glp-4(bn2ts);sek-1(km4)* worms were infected for 8 h on lawns of MMH594 as described above. The worms were resuspended in media composed of 20% BHI, 80 $\mu\text{g}/\text{ml}$ kanamycin, and 80% M9 buffer. Approximately 25 worms in a volume of 50 μl were transferred into 0.3-ml wells of 96-well plates. An equal volume of liquid media containing 125 units/ml nystatin and the compounds or extracts to be tested were mixed into the wells. Each compound or extract was tested in individual wells, and the screen was performed by using duplicate 96-well plates. The final concentration of compounds from ChemBridge Diverset E was 25 $\mu\text{g}/\text{ml}$ with DMSO at 1%. The concentration of the extracts from the natural product NCI library was 150 or 200 $\mu\text{g}/\text{ml}$ with DMSO at 1.5 or 2%. The plates were sealed with gas-permeable membranes (Breath-easy, Diversified Biotech) and incubated without agitation at 26°C and 80–85% relative humidity.

Each 96-well plate contained 80 test compounds that were tested with worms infected with the cytolytic-positive *E. faecalis* strain MMH594. The remaining 16 wells contained positive and negative

controls to determine whether the assay yielded predictable and reproducible responses to antibiotics or avirulent *E. faecalis* mutants and a clear threshold between positive and negative responses. Each plate contained eight negative control wells that did not contain any antimicrobial compound and four positive control wells that contained 20 $\mu\text{g}/\text{ml}$ ampicillin (two wells) or 20 $\mu\text{g}/\text{ml}$ tetracycline (two wells). These control wells were seeded with *glp-4;sek-1* infected with *E. faecalis* MMH594. In addition, each plate contained four positive control wells that were seeded with *glp-4;sek-1* worms infected with the *E. faecalis* ΔfsrB mutant. For the screen of 1136 NCI extracts, each 96-well plate contained 88 samples, four negative control wells, two positive control wells that contained 20 $\mu\text{g}/\text{ml}$ ampicillin or 20 $\mu\text{g}/\text{ml}$ tetracycline, and two positive control wells seeded with worms infected with the ΔfsrB mutant. In total, there were 1,312 control wells in the ChemBridge and NCI screens. The false-positive rate was 2.7%, and the false-negative rate was 1.9%. False positives were defined as $>50\%$ survival of infected worms in wells that did not contain an antibiotic. False negatives were defined as $<50\%$ survival of worms treated with an antibiotic or infected with the ΔfsrB mutant.

Worm survival was scored manually after 6 days of incubation. A total of 6,000 compounds from a ChemBridge library Diverset E and 1,136 extracts from the NCI Natural Products Repository (www.dtp.nci.nih.gov/branches/npb/repository.html) were screened. Compounds or extracts that increased worm survival by 2- to 3-fold were retested for activity. MICs were determined against *E. faecalis* strain MMH594 by using 2-fold dilution in BHI media according to the broth microdilution protocol of the Clinical and Laboratory Standards Institute [CLSI; formerly National Committee for Clinical Laboratory Standards (NCCLS)] (40). The ability of compounds to hemolyze sheep erythrocytes was based on the protocol of Ciernei *et al.* (41), with the following modifications: sheep erythrocytes (Rockland Immunochemicals) were treated with compounds (100 $\mu\text{g}/\text{ml}$) in PBS with DMSO at 2% for 2 h and the supernatants were monitored at OD₅₄₀.

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