Automated Laboratory Growth Assessment and Maintenance of *Azotobacter vinelandii*

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Azotobacter vinelandii (*A. vinelandii*) is a commonly used model organism for the study of aerobic respiration, the bacterial production of several industrially relevant compounds, and, perhaps most significantly, the genetics and biochemistry of biological nitrogen fixation. Laboratory growth assessments of *A. vinelandii* are useful for evaluating the impact of environmental and genetic modifications on physiological properties, including diazotrophy. However, researchers typically rely on manual growth methods that are oftentimes laborious and inefficient. We present a protocol for the automated growth assessment of *A. vinelandii* on a microplate reader, particularly well-suited for studies of diazotrophic growth. We discuss common pitfalls and strategies for protocol optimization, and demonstrate the protocol's application toward growth evaluation of strains carrying modifications to nitrogen-fixation genes. © 2021 The Authors.

Basic Protocol 1: Preparation of *A. vinelandii* plate cultures from frozen stock
Basic Protocol 2: Preparation of A. *vinelandii* liquid precultures
Basic Protocol 3: Automated growth rate experiment of *A. vinelandii* on a microplate reader

Keywords: *Azotobacter vinelandii* • bacterial growth • microplate reader • nitrogen fixation • nitrogenase

INTRODUCTION

Azotobacter vinelandii (*A. vinelandii*), a Gram-negative, obligately aerobic, diazotrophic Gamma proteobacterium, has been an important model organism since its discovery more than a century ago (Lipman, 1903). One of the most significant contributions of the *A. vinelandii* model has been toward understanding the evolution, genetics, and biochemistry of nitrogenase-catalyzed nitrogen fixation (Dos Santos, 2019; Hoffman, Lukoyanov, Yang, Dean, & Seefeldt, 2014; Hu, Fay, Lee, Wiig, & Ribbe, 2010; Noar & Bruno-Barcena, 2018). *A. vinelandii* growth assays are utilized to evaluate the physiological
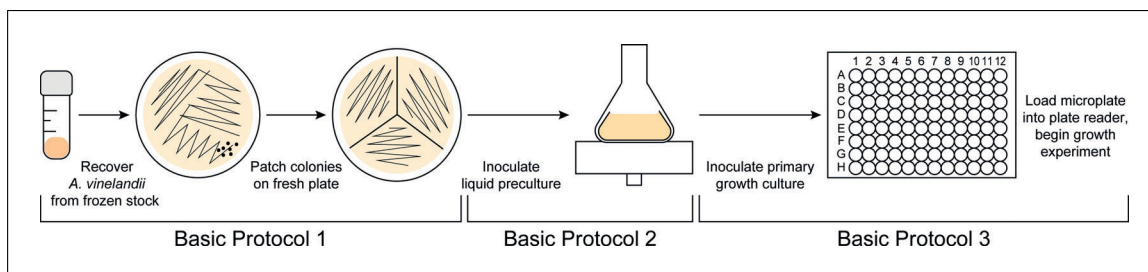
Figure 1 Basic protocols for automated growth assessment of *A. vinelandii*. *A. vinelandii* cells are recovered from frozen stock (Basic Protocol 1) and used to prepare a liquid preculture inoculum (Basic Protocol 2). Growth medium is inoculated with the preculture, aliquoted into plate wells, and loaded into a microplate reader for optical density measurements over the growth period (Basic Protocol 3). For assessments of diazotrophic growth, precultures are prepared in Burk's medium supplemented with nitrogen, and used to inoculate nitrogen-free Burk's medium for the primary growth experiment.

impact of genomic modifications and varied environmental conditions on diazotrophic growth (Arragain et al., 2017; McRose, Baars, Morel, & Kraepiel, 2017; Mus, Tseng, Dixon, & Peters, 2017; Plunkett, Knutson, & Barney, 2020). However, manual assays of *A. vinelandii*, which have a doubling time of ∼2 to 4 hr under normal diazotrophic conditions (Page & von Tigerstrom, 1979), are typically laborious and time consuming. Moreover, automated growth methods that are common for other model bacteria such as *Escherichia coli* (Hall, Acar, Nandipati, & Barlow, 2014; Kurokawa & Ying, 2017; Wong, Mancuso, Kiriakov, Bashor, & Khalil, 2018) are not yet reported for *A. vinelandii*.

Addressing this gap, here we present a laboratory protocol for high-throughput *A. vinelandii* growth evaluation on a microplate reader, which can provide precise measurements of growth rates that are comparable or preferable to those obtained by manual spectrophotometer measurements (Hall et al., 2014). This protocol details *A. vinelandii* growth assessments beginning with recovery of bacterial stocks and preparation of inoculum for liquid cultures, and provides instruction for culture maintenance and automated optical density measurements on a standard microplate reader (Fig. 1). This approach is appropriate for the efficient investigation of growth rates for wild-type and engineered *A. vinelandii* strains, as well as for their evaluation under varying physical and nutritional environmental conditions of interest.

CAUTION: Azotobacter vinelandii is a Biosafety Level 1 (BSL-1) organism. Such organisms are not known to consistently cause disease in healthy adult humans and are of minimal potential hazard to laboratory personnel and the environment. Standard microbiological practices should be followed when working with these organisms. See Burnett, Lunn, & Coico, 2009 for more information.

STRATEGIC PLANNING

Strain Acquisition

The laboratory wild-type variant of *A. vinelandii* is the non-alginate-producing OP strain (Burk & Lineweaver, 1930; Dos Santos, 2019), a spontaneous mutant of the alginate-producing strain isolated by (Lipman, 1903). Alternative designations of the OP strain include the UW (University of Washington) and CA (North Carolina State University) strains. Nevertheless, several non-wild-type *A. vinelandii* variants including alginate-producing strains are commonly used for different applications. Some of these variants are available from the American Type Culture Collection (ATCC), including the high-frequency-transforming DJ strain, the genome of which has been fully sequenced (Setubal et al., 2009) (ATCC BAA-1303). Due to the availability of genomic sequence information and tractability for genetic manipulation studies, the DJ strain was selected for development of this protocol, generously provided by Dennis Dean (Virginia Tech). However, other strains may be used depending on the desired application.
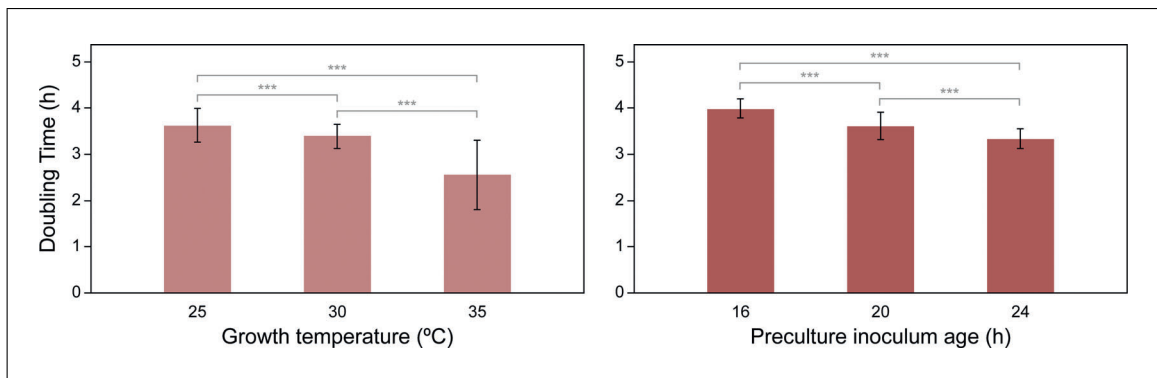
Figure 2 Mean *A. vinelandii* DJ diazotrophic doubling times measured grown across a range of temperatures (left) and from preculture inocula of varying ages (right). Mean doubling times calculated across three experimental trials. Error bars represent 1σ. Asterisks indicate *p*-values ≤ 0.0001 calculated from a post-hoc Tukey's HSD test following a one-way ANOVA.

Optimization of growth conditions

Microplate readers with temperature control and continuous shaking capabilities can be used for *A. vinelandii* maintenance and growth rate determination. Standard *A. vinelandii* cultures are typically grown in flasks and shaken from 100 to 300 rpm at 30°C (Arragain et al., 2017; Dos Santos, 2019; McRose et al., 2017; Mus et al., 2017). However, these growth conditions can be further optimized or varied depending on experimental design and instrument capabilities.

Factors that may impact growth and should, thus, be considered when designing the experiment include temperature, shaking speed, culture volume, and inoculum preparation. As an example, we assessed *A. vinelandii* growth as a function of variation in temperature and preculture inoculum age (Fig. 2). We found that both temperature and preculture inoculum age led to significant ($p < 0.05$) differences in doubling times, with both increasing temperature and increasing inoculum age resulting in faster doubling times across the measured range. It is recommended that the user similarly optimize for key growth conditions with their equipment. Culture volume in particular may have a significant effect on growth rate by altering the amount of headspace in the well and culture movement during shaking. Culture volume should be optimized after shaking speed, as different volumes may be optimal at different shaking speeds. Additional care should be given to ensure consistency across the plate wells, which can be accomplished by using a lid or gas-permeable membrane to minimize evaporation and cross-contamination. The use of these items may result in slower *A. vinelandii* growth rates as compared to flask-grown cultures due to impeded aeration but should not preclude comparisons across samples grown in similar conditions.

Reproducibility

All qualitative and quantitative experimental parameters, particularly those used to operate the microplate reader, should be reported to facilitate future reproducibility (Chavez, Ho, & Tan, 2017). Multiple repeat measurements can be made simultaneously across aliquots of the same samples in a single microplate, but experimental replicates should be performed on different inoculations and days to account for variability of the bacterial stock and day-to-day instrument performance.

Data analysis

When analyzing growth measurements from high-throughput procedures, it is recommended to use reproducible and efficient data analysis methods. Several free software packages are available for analysis of growth data. The R package GrowthCurver (Sprouffske & Wagner, 2016) was used for analyses reported in this protocol.

GrowthCurver calculates growth parameters by fitting data to a standard form of the logistic equation and is well suited for handling high-throughput growth measurements.

PREPARATION OF *A. VINELANDII* PLATE CULTURES FROM FROZEN STOCKS

A growth rate experiment of *A. vinelandii* begins with strain recovery from frozen stocks. *A. vinelandii* stocks are typically kept in a 7% DMSO storage buffer and stored at –80°C. Frozen cells can then be directly streaked on solid medium to prepare isogenic plate cultures for subsequent growth rate assessments.

A. vinelandii is generally grown in Burk's medium (B medium; see Reagents and Solutions), which may be supplemented with a nitrogen source (BN medium), antibiotics, or modified trace metals as desired. This protocol describes recovery on BN plates (rather than B plates) so that cells can be grown regardless of the diazotrophic capabilities of the strain, which can later be assessed during the growth rate experiment. If needed, recovery can also be accomplished with BN plates supplemented with antibiotics.

Materials

A. vinelandii (ATCC BAA-1303) frozen stock on ice
BN solid medium plates (see recipe)

Bunsen burner
Biosafety cabinet (optional)
Inoculation loops or needles (sterile)
30°C incubator
4°C refrigerator (as needed)
−80°C freezer

1. Use a sterile inoculation loop or needle to streak a small amount (enough to be just visible on the tip of the inoculation tool) of frozen cells onto a BN plate. Streak for isolation, and immediately return frozen stock to –80°C.

   Follow proper aseptic technique to minimize risk of contamination. Work should be performed near a Bunsen burner or in a biosafety cabinet.

2. Incubate plates for 2 to 3 days at 30°C.

   To prevent plates from drying, keep a small beaker of sterile water in the incubator or seal the plate with parafilm. Cells plated on BN medium typically range from greenish yellow (when dense) to cream in color. Plates can be sealed with parafilm and stored up to 1 month at 4°C.

3. Use a sterile inoculation loop or needle to patch one full-sized colony onto a fresh BN plate.

   Patch one colony for each planned growth experiment replicate to account for potential genetic variation within the bacterial stock. BN plates should be split into four patched sections at most to ensure sufficient cells for subsequent inoculation into liquid medium.

4. Incubate plates for 2 to 3 days at 30°C.

   Use patched cells for growth rate experiments as soon as possible, and no more than a week after storage at 4°C.

PREPARATION OF *A. VINELANDII* LIQUID PRECULTURES

Preparation of *A. vinelandii* liquid precultures prior to the growth rate experiment is necessary to produce inocula that are in a consistent physiological state across different

strains and replicates. The age of the preculture at the time of inoculation into the primary growth culture can be optimized as needed. A saturated preculture tends to provide a longer and more easily detectable lag phase for curve fitting in downstream growth rate analyses. Furthermore, it minimizes the volume of preculture needed to inoculate the primary culture, which can introduce undesirable residual medium additions (such as supplemental nitrogen) from the preculture that result in a variable lag phase.

Materials

Isogenic *A. vinelandii* plate culture (from Basic Protocol 1)
BN medium (sterile; see recipe)

Glass 125-ml flasks capped with aluminum foil (sterile)
Serological pipets (sterile)
Inoculation loops or needles (sterile)
Vortex (optional)
30°C shaking incubator

1. Using a serological pipet, transfer 50 ml sterile, room temperature BN liquid medium into a 125-ml glass flask.

   Follow proper aseptic technique. Work should be performed near a Bunsen burner or in a biosafety cabinet. Other medium volumes and flask sizes can be used, as long as there is sufficient aeration during shaking. 50 ml medium will provide more than enough material for subsequent inoculation of the main growth culture. Antibiotics can be added here or later in the main culture if needed.

2. Use a sterile inoculation loop or needle to inoculate the BN liquid medium with *A. vinelandii* cells (full ~1 to 5 μl loop). Let cells sit in liquid medium for ~5 min, then swirl or vortex to completely resuspend.

3. Shake liquid culture for 24 hr at 30°C and 300 rpm.

   See Basic Protocol 2 introduction for discussion of preculture age. Saturated cultures in BN medium are typically light cream in color but can be tinged green depending on the strain and/or growth conditions (see Fig. 3).

AUTOMATED GROWTH RATE EXPERIMENT OF *A. VINELANDII* ON A MICROPLATE READER

This protocol focuses on the automated assessment of *A. vinelandii* diazotrophic growth in B (nitrogen-free) medium on a microplate reader. Liquid precultures grown in BN (Basic Protocol 2) are used to inoculate B medium to a desired starting optical density at 600 nm (OD_{600}) for the growth rate experiment. In B medium, *A. vinelandii* quickly use up any residual ammonia from the preculture and begin expressing nitrogenase genes for nitrogen fixation. Detected growth is, thus, directly related to the strain's ability to express nitrogenase and grow diazotrophically. Experiments targeting other aspects of *A. vinelandii* physiology can be designed by modifying the preculture and primary culture medium with, for example, antibiotics or different concentrations of trace metals. Over the course of the growth rate experiment, the microplate reader can provide automated temperature control, shaking, and optical density measurements, all parameters which can be varied to test different growth conditions as permitted by the instrument capabilities.

Materials

A. vinelandii saturated preculture in BN medium (from Basic Protocol 2)
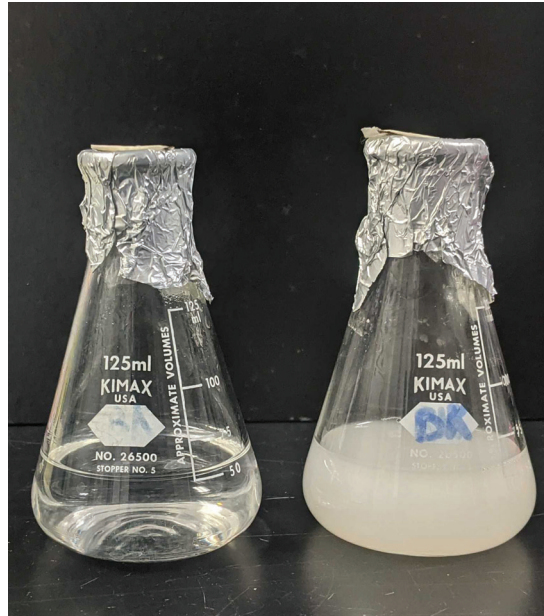B medium (sterile; see recipe)

Figure 3 *A. vinelandii* DJ liquid culture in BN media after 24 hr of growth at 30°C shaken at 300 rpm (right) and the BN control (left).

Glass flasks (sterile)
Serological pipets (sterile)
96-well microplates, clear, flat-bottomed (sterile) (Greiner Bio-One, cat. no. 655161)
Multichannel pipette
50-ml pipetting reservoirs (sterile)
Microplate lid (optional) (Greiner Bio-One, cat. no. 656171)
Gas-permeable microplate membrane (optional) (Sigma-Aldrich, cat. no. Z380059)
Microplate reader (Tecan Spark, cat. no. 30086376)

Prepare the primary growth culture

1. In a sterile glass flask (or other small vessel), prepare a 1:10 dilution of the *A. vinelandii* preculture in sterile B medium (e.g., 1 ml preculture in 9 ml B medium). Swirl gently to mix.

   Follow proper aseptic technique. Work should be performed near a Bunsen burner or in a biosafety cabinet.

2. Transfer 125 µl of the diluted *A. vinelandii* preculture to a single well of a 96-well microplate. In addition, transfer 125 µl of sterile B medium to a separate well to serve as blank reference for the absorbance reading.

   The sample volume in the microplate well determines the length of the light path, and thus affects absorbance readings. The volume used for OD_{600} measurement of the diluted preculture should also be used for the growth rate experiment, including blank wells.

   Sample volume should be optimized beforehand (see Strategic Planning), particularly if alternate microplate formats (e.g., 384-well microplates) are used.

3. Measure the OD_{600} of the diluted preculture on the microplate reader, taking a single absorbance reading of the wells.

OD$_{600}$ measurement of the initial diluted preculture should be performed using the same microplate reader that will be used for the growth rate experiment. This removes the potential for measurement variation across different spectrophotometers and ensures that the investigator accurately prepares the primary growth culture to the desired optical density. Zeroing using the blank reference can be performed automatically by the plate reader, or the blank absorbance reading can simply be subtracted from the preculture reading to determine the actual OD$_{600}$ of the sample.

4. Based on the initial OD$_{600}$ value of the diluted preculture, in a glass flask (or other appropriate vessel), inoculate B medium to obtain a final OD$_{600}$ of ∼0.05 for the main growth culture. Swirl gently to mix.

   Prepare a final volume that can sufficiently be aliquoted across the desired number of wells for the growth rate experiment. Note that excess volume may be needed to comfortably pipet from a reagent reservoir in the following step (e.g., for 125 μl across 96 wells, prepare >20 ml). Antibiotics can be added to the B medium prior to inoculation if desired.

Prepare microplate and perform growth rate experiment

5. Pour the primary growth culture into a reagent reservoir. Use a multichannel pipette to aliquot 125 μl across the wells of a microplate. Reserve at least one well to fill with 125 μl of B medium to serve as a blank reference for the experiment.

6. Use a lid or gas-permeable membrane to cover the wells of the microplate.

   If using a lid, note that significant evaporation can still occur for wells close to the edge of the microplate and it may be preferable to leave these wells empty (Chavez et al., 2017; Hall et al., 2014). If using a membrane, take care to ensure that there are minimal creases or bubbles when applied. Improper membrane application may lead to inconsistent growth rates across the microplate because these surface area differences influence aeration.

7. Insert the prepared microplate into the reader, set the instrument parameters, and start the growth experiment program.

   Microplate reader parameters that should be considered include:

   - Temperature (typically 30°C)
   - Shaking speed (typically ∼300 rpm)
   - Shaking mode (e.g., orbital)
   - Measurement interval (e.g., once per hour)
   - Experiment duration (see below)
   - Absorbance wavelength (typically 600 nm) and other measurement parameters

   (e.g., single vs. multiple reads per well)

   These parameters should be optimized beforehand (see Strategic Planning). A. vinelandii cultures grown on a microplate reader typically reach saturation after ∼48 hr. However, growth should be monitored longer (up to ∼72 hr) to ensure sufficient duration of the stationary phase for curve fitting in downstream analyses. The appropriate experimental duration for mutant strains or modified growth conditions may vary.

8. Save the growth experiment file and export for subsequent data analysis (see Strategic Planning and Fig. 4).

REAGENTS AND SOLUTIONS

Ammonium acetate solution, 100×

   Weigh 10 g ammonium acetate (Sigma-Aldrich, cat. no. A7262) into a 250-ml beaker
   Dissolve in dH$_2$O up to 100 ml
   Filter sterilize (0.2 μm) 10-ml aliquots
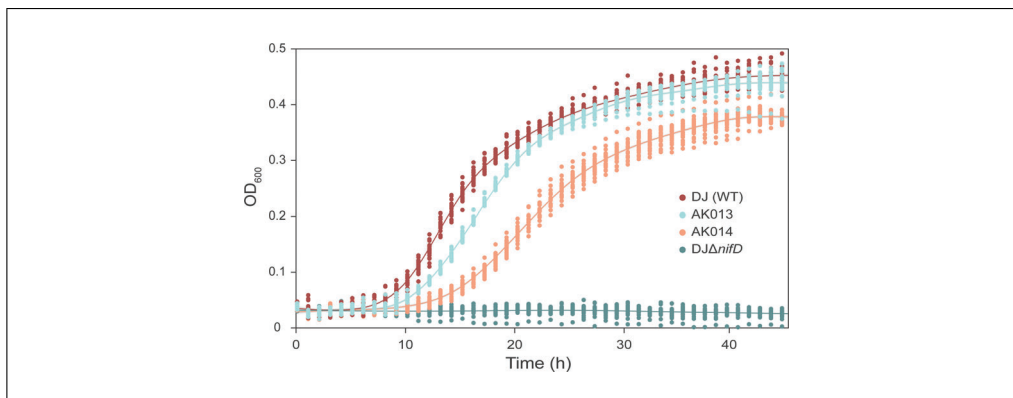   Store up to 1 year at room temperature

Figure 4 Diazotrophic growth curves for wild-type (WT) and engineered *A. vinelandii* strains. Optical density at 600 nm (OD_{600}) measured once per hour for multiple wells in a single experimental trial. Mean growth curve for each strain indicated by solid line.

Burk's (B) medium/Burk's nitrogen-supplemented (BN) medium

Dilute 5 ml sterile $10\times$ salts solution into 45 ml sterile $1\times$ phosphate buffer
Optional for BN medium: Add 0.5 ml sterile $100\times$ ammonium acetate solution
Prepare fresh

Burk's (B) medium/Burk's nitrogen-supplemented (BN) medium, solid

Weigh 8 g agar (Sigma-Aldrich, cat. no. A1296) into a 1-L bottle
Dilute 4.5 ml $100\times$ phosphate buffer into 445 ml dH_2O
Sterilize by autoclaving for 20 min at 121°C
Cool to 55°C in a water bath
Add 50 ml sterile $10\times$ salts solution
Optional for BN medium: Add 5 ml sterile $100\times$ ammonium acetate solution
Pour ~25 ml into plates and cool
Store plates in sealed sleeve up to several months at 4°C

Dimethyl sulfoxide (DMSO) storage buffer, 7%

Dilute 7 ml DMSO (Sigma-Aldrich, cat. no. 472301) into 90 ml sterile $1\times$ phosphate buffer
Mix well (do not filter sterilize)
Divide into 1-ml aliquots into cryovials for strain storage
Store up to 2 days at 4°C

Phosphate buffer, 100×

Weigh 22 g KH_2PO_4 (Sigma-Aldrich, cat. no. P5379) and 88 g K_2HPO_4 (Sigma-Aldrich, cat. no. P8281) into a 1-L bottle
Dissolve in dH_2O up to 1 L
Store up to 1 year at room temperature

Phosphate buffer, 1×

Dilute 5 ml $100\times$ phosphate buffer into 495 ml dH_2O
Sterilize by autoclaving for 20 min at 121°C
Store up to 1 year at room temperature

Salts solution, 10×

Add 100 g sucrose (Sigma-Aldrich, cat. no. S0389), 1 g $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, cat. no. M2773), 0.45 g $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, cat. no. 223506), 0.5 ml of 10 mM $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich, cat. no. 331058), and 25 mg $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich, cat. no. F8633) to 1-L bottle

Dissolve in dH$_2$O up to 500 ml
Sterilize by autoclaving for 20 min at 121°C
Store up to 1 year at room temperature

COMMENTARY

Background Information

Since its discovery more than 100 years ago (Lipman, 1903), *A. vinelandii* has served a variety of scientific and industrial applications. These include the study of aerobic respiration (Poole & Hill, 1997), hydrogen uptake and production (Kow & Burris, 1984; Noar, Loveless, Navarro-Herrero, Olson, & Bruno-Barcena, 2015), polymer production (Clementi, 1997; Galindo, Pena, Nunez, Segura, & Espin, 2007), and, importantly, the genetics and biochemistry of nitrogenase-catalyzed nitrogen fixation (Dos Santos, 2019; Hoffman et al., 2014; Noar & Bruno-Barcena, 2018). Due to the challenge of heterologous expression of functional nitrogenases *A. vinelandii* has been used for their expression, purification, and biochemical characterization (Hoffman et al., 2014; Lee, Ribbe, & Hu, 2019; Solomon et al., 2020). Furthermore, *A. vinelandii,* which exhibits diauxic metabolism (George, Costenbader, & Melton, 1985) and grows in a wide range of oxygen levels (Dingler, Kuhla, Wassink, & Oelze, 1988), is an important model for studying nitrogen fixation in diverse environments. Recently, this organism has been involved in a renewed interest in biofertilizers (Ambrosio, Ortiz-Marquez, & Curatti, 2017; Khdhiri et al., 2017; Nosheen et al., 2016; Olivares, Bedmar, & Sanjuan, 2013), and the examination and manipulation of nitrogenase genes in *A. vinelandii* have informed efforts to engineer nitrogen fixation in cereal crops to improve agricultural yields (Buren & Rubio, 2018; Jimenez-Vicente & Dean, 2017). Finally, *A. vinelandii* serves as a promising microbial background for the insertion of reconstructed ancestral nitrogenase genes, which can reveal phenotypic insights into early evolutionary states of nitrogen fixation (Garcia & Kacar, 2019).

The aerobic *A. vinelandii* is an appealing model organism for laboratory work due to its relative ease of maintenance, genetic tractability, and non-pathogenicity (Biosafety Level 1), as compared with other pathogenic or anaerobically cultivated diazotroph models (e.g., *Clostridium pasteurianum*, *Klebsiella pneumoniae*, *Rhodopseudomonas palustris*). *A. vinelandii* can be maintained in ambient, benchtop conditions, though growth is typically optimized and standardized in an incubator. Natural competency for transformation in *A. vinelandii* can be induced via metal starvation and visually confirmed due to the associated production of fluorescent green siderophores (McRose et al., 2017). This characteristic makes it a highly suitable organism for genetic manipulation, as reviewed in (Dos Santos, 2019). Growth rate assessments of genetically modified *A. vinelandii* can, therefore, reveal the physiological contributions of nitrogenase and nitrogenase-related genes (Arragain et al., 2017; Garcia, McShea, Kolaczkowski, & Kaçar, 2020; McRose et al., 2017; Mus, Colman, Peters, & Boyd, 2019; Plunkett et al., 2020).

Critical Parameters and Troubleshooting

It is important to optimize conditions for *A. vinelandii* growth on a microplate reader prior to growth rate assessment to ensure consistency and reproducibility across biological and technical replicates (see Strategic Planning). Growth conditions that should be considered include temperature, shaking speed, culture volume, and inoculum preparation.

A common problem encountered during growth rate assessment on a microplate reader (Basic Protocol 3) is well evaporation over the approximately 48 to 72 hr needed for *A. vinelandii* cultures to reach saturation. Evaporation can be minimized by use of a lid or gas-permeable membrane. However, significant evaporation can still occur at plate edges when using a lid (Chavez et al., 2017), and improper application of the membrane can result in variable growth rates across the plate. Since such variability is challenging to eliminate entirely, it is important to include several technical replicates across the plate and avoid measuring wells at the plate edges when using a lid.

Time Considerations

The initial recovery of *Azotobacter* strains (Basic Protocol 1) and preparation of isogenic plate cultures (Basic Protocol 2) takes approximately 6 days. Preculture preparation (Basic Protocol 2) takes approximately 24 hr. This time can be optimized but should be made consistent across replicates. Each microplate reader growth experiment takes approximately

Table 1 Mean Diazotrophic Doubling Times for Wild-Type and Engineered *A. vinelandii* Strains. No Diazotrophic Growth Detected for DJΔ*nif*D

	Mean doubling time $\pm$ 1σ (hr)		
Strain	DJ (wild-type)	AK013	AK014
Trial 1	2.95 $\pm$ 0.13	2.83 $\pm$ 0.11	3.62 $\pm$ 0.20
Trial 2	2.88 $\pm$ 0.17	2.74 $\pm$ 0.11	3.47 $\pm$ 0.30
Trial 3	4.63 $\pm$ 0.18	3.23 $\pm$ 0.10	4.36 $\pm$ 0.24

48 to 72 hr, though this time may vary depending on the tested strain and growth conditions. The experiment should be maintained until cultures reach saturation to ensure reliable curve-fitting during subsequent growth data analysis.

Understanding results

This protocol can also be used to compare the growth rates and characteristics of different wild-type and engineered *A. vinelandii* strains, as well as different physical and nutritional growth conditions. To provide an example of anticipated results for the protocol described here, we conducted diazotrophic growth rate experiments on wild-type *Azotobacter vinelandii* DJ and three strains harboring modifications to the nitrogenase *nifD* gene. The *nifD* gene encodes the active site-containing subunit of the nitrogenase enzyme. Modifications to this gene are expected to influence nitrogenase N_2-reduction and, thus, the ability of *A. vinelandii* to grow diazotrophically. The modified strains include "AK013" and "AK014", which have 93% and 81% *nifD* DNA identity to *nifD* of the wild-type DJ strain, respectively, and a DJΔ*nifD* deletion strain.

Figure 4 shows growth curves for each *A. vinelandii* strain, and Table 1 reports mean doubling times calculated with the R package GrowthCurver (Sprouffske & Wagner, 2016). We did not detect diazotrophic growth for DJΔ*nifD*. For Trials 1 and 2, AK014 grew slower than both DJ and AK013 ($p < 0.05$; calculated from a post-hoc Tukey's HSD test following a one-way ANOVA), but no difference in doubling times was found for DJ and AK013. However, for Trial 3, DJ grew significantly slower than both AK013 and AK014. This result highlights the need to repeat growth experiments on multiple days to account for day-to-day instrument variability. This automated protocol for evaluating diazotrophic growth differences across different *A. vinelandii* strains can be adapted for a variety of additional applications.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

Brooke M. Carruthers: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; writing-original draft; writing-review & editing. **Amanda K. Garcia:** Data curation; Formal analysis; Investigation; Methodology; Supervision; writing-original draft; writing-review & editing. **Alex Rivier:** Data curation; Formal analysis; Investigation; Validation; writing-review & editing. **Betul Kacar:** Conceptualization; Investigation; Methodology; Project administration; Resources; Supervision; writing-review & editing.

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