Azotobacter vinelandii: the source of 100 years of discoveries and many more to come

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Abstract
Azotobacter vinelandii has been studied for over 100 years since its discovery as an aerobic nitrogen-fixing organism. This species has proved useful for the study of many different biological systems, including enzyme kinetics and the genetic code. It has been especially useful in working out the structures and mechanisms of different nitrogenase enzymes, how they can function in oxic environments and the interactions of nitrogen fixation with other aspects of metabolism. Interest in studying A. vinelandii has waned in recent decades, but this bacterium still possesses great potential for new discoveries in many fields and commercial applications. The species is of interest for research because of its genetic pliability and natural competence. Its features of particular interest to industry are its ability to produce multiple valuable polymers – bioplastic and alginate in particular; its nitrogen-fixing prowess, which could reduce the need for synthetic fertilizer in agriculture and industrial fermentations, via coculture; its production of potentially useful enzymes and metabolic pathways; and even its biofuel production abilities. This review summarizes the history and potential for future research using this versatile microbe.

INTRODUCTION
Soon after the discovery of Azotobacter vinelandii in 1903 [1], it was adopted as a model organism for the study of aerobic nitrogen fixation, respiration, microbial physiology, hydrogen production and assimilation, and other enzyme kinetics. Multiple important discoveries have been made studying azotobacters, including Lineweaver–Burk kinetic models and the genetic code [2, 3]. The genus’s features of most interest, however, have been its nitrogenases and nitrogen-fixing capabilities [4–6] and aerobic physiology [7]; its respiration rates are some of the highest observed [8–10].

Interest in studying these microbes has waned since the early 1970s (Fig. 1), but they may be due for a comeback. Effort is being made to sequence Azotobacter genomes [11–13] and tap into their biotechnological and agricultural potential [14–18]. This review aims to revisit the discoveries of the past and lay out the prospects for discoveries in the future, including the study of hydrogen production that will be the focus of later sections.

GENERAL CHARACTERISTICS OF AZOTOBACTER VINELANDII
There is some suggestion that the genus Azotobacter should be subsumed by the genus Pseudomonas, based mainly on sequence similarities and alginate production, although different phylogenies give differing results [11, 19–22]. The genus’s chief characteristics include obligate aerobic lifestyles [23, 24], diazotrophy [1, 8, 25, 26], formation of desiccation-resistant cysts [27], production of polyhydroxalkanoates (PHAs) as storage polymers [28], and natural competence [29–31]. Wild-type strains are often motile [1, 8], and cell morphology is pleomorphic, varying depending on physiological state and growth conditions [8, 32–34].

A. vinelandii was isolated first from soil in Vineland, New Jersey [1]. It possesses three versions of nitrogenase with different metals in their central cofactors [25, 26, 35–38]. These enzymes can substitute one for another to allow growth when one or another essential metal is absent [39–41], or at different temperatures [42]. Growing in iron-limited conditions, the species produces a characteristic neon-green pigment, a diffusible siderophore called azotobactin [43–45]; it also produces several other siderophore compounds [44, 46]. A. vinelandii can use a great variety of carbon substrates, including alcohols, organic acids and sugars [8, 47], although it displays a preference for certain compounds over others in the form of diauxic growth [48–50]. This versatility raises the possibility of low-cost, unrefined carbon substrates for biotechnological processes [49, 51, 52].
As a model organism for research, A. vinelandii also displays several favourable characteristics. It is naturally competent in certain conditions: it takes up linear DNA and performs double recombination with homologous portions of its genome, permitting stable modifications [29–31, 53, 54]. The optimal conditions for transformation depend on a given strain’s characteristics, especially alginate-producing ability, but generally competence is highest in late exponential phase under iron limitation, with sugars or glycerol rather than organic acids as a carbon source [29, 30]. Gummy (alginate-producing) strains of A. vinelandii are generally less competent than non-gummy strains [29, 53], presumably because alginate acts as a barrier between the cells and exogenous DNA, but competence in such strains can be enhanced, such as by replacing glucose with other carbon substrates (especially sugar alcohols) [53], by increasing phosphate or decreasing calcium concentration [29] or by adding cAMP [54]. In non-gummy strains, such as A. vinelandii strain CA, molybdenum limitation and excess magnesium enhance competence [29, 31, 53, 54]. When transformation is performed in liquid rather than on solid medium, adding a limited amount of fixed nitrogen is necessary [30]. Competent cells have been observed to contain greater amounts of poly-β-hydroxybutyrate (PHB) than non-competent cells, but it is not clear whether competence and PHB are causally related [55]. Such competence enhances the value of A. vinelandii as a research and biotechnology organism, permitting straightforward gene knockouts, mutations, and introduction of new traits in the organism. A strategy has even been developed for markerless genetic modification [56].

There has been some concern that A. vinelandii cells may be polyploid, containing as many as 80 copies of their genome per cell [57, 58]; this could complicate transformation, making it difficult to obtain homozygous transformants, at least at certain loci [57]. However, others have found that segregation behaviour in A. vinelandii does not appear consistent with polyploidy [59–61]. A possible explanation is that in rich medium, freshly divided cells have one or a few copies of their chromosome, while cells in late-exponential or stationary phase have 10- to 50-fold more [58]. This effect is not observed in minimal media [58]. Therefore, this effect should not be an insurmountable obstacle to transformation.

In addition to linear DNA, A. vinelandii can maintain and be transformed with plasmids of certain kinds [62–67]. Plasmids of the IncQ and IncP-1 groups have been successfully transformed into and maintained in A. vinelandii using conjugation from Escherichia coli [64, 68], electroporation [63, 67], and the natural competence method of Page and von Tigerstrom discussed previously [62]. Plasmids can also be used to introduce transposons into A. vinelandii for mutagenesis [68–70]. These possibilities further enhance the organism’s usefulness.

**HISTORICAL INTEREST**

**Nitrogen fixation**

Since its discovery as a nitrogen-fixing soil microbe in 1903, A. vinelandii has been studied throughout the decades as an aerobic, diazotrophic model organism [1, 71–73]. Its largest contribution, from discovery to the present day, has been to the study of enzymes relating to nitrogen fixation. As mentioned, A. vinelandii has three separate versions of nitrogenase [25, 26, 35–38, 74], one of which contains an atom of molybdenum (Mo), one with vanadium (V) in the place of Mo, and a third with iron (Fe) in place of the heterometal. They all reduce N₂ to NH₃ but have minor differences from each other. They each have two main components: dinitrogen reductase (DNR) and dinitrogenase [75, 76]. The former is a single subunit encoded by one gene and the latter comprises at least two subunits (α and β) that form an α₂β₃ tetramer in the Mo nitrogenase [76, 77], while the V and Fe nitrogenases also contain a δ subunit [78, 79]. Some studies have investigated the specific amino acid residues essential for the catalysis, especially in the Mo nitrogenase [80, 81]. Other necessary enzymes are not involved in catalysing the reaction, but rather assembling the cofactors and inserting them into the apoenzymes [74, 82, 83]. The nitrogenase as a whole is located in the cytoplasm, not associated with the cell membrane [73], and makes up about 10 % of the total cell protein when cells are fixing nitrogen [84].

The process of dinitrogen reduction begins when the DNR consumes two ATP molecules required for the reaction [85, 86]. This is the rate-limiting step in the reaction [87], allowing the transfer of an electron from an electron carrier such as flavodoxin [88, 89] to the DNR, thereby ‘charging’ it [86, 90]. This electron is then transferred from the DNR to the dinitrogenase, where a molecule of N₂ binds [75, 90]. This process repeats at least seven more times (in the case of the Mo nitrogenase), resulting in the addition of six electrons and protons to the N₂ molecule and the reduction of two protons to a molecule of H₂ [91]. Nitrogenases can also reduce other substrates, in place of or in competition with dinitrogen and protons. For example, carbon monoxide (CO) is reduced to ethylene and other hydrocarbons,
especially by the V nitrogenase [92–94], although this reaction does not contribute to the cell’s primary carbon metabolism [95]. Other substrates include acetylene (C2H2), azide (N3⁻) and cyanide (CN⁻). Carbon dioxide can be reduced to methane and other hydrocarbons [96, 97], and the Fe proteins can interconvert CO2 and CO, similar to carbon monoxide dehydrogenase [98]. The ratio of nitrogenase components (DNR and dinitrogenase) affects the rate of electron flow and the proportion of electrons that go to a given substrate when multiple are available [75, 99, 100]; the average ratio of dinitrogenase to DNR in vivo is 1 to 1.45 [84, 88]. As delicate redox catalysts, however, these enzymes are sensitive to inactivation by various reactive chemicals. The DNR can be irreversibly inactivated by oxygen exposure [101], and although dinitrogenase can reduce CO, azide, N2, O and cyanide to some degree, these compounds inhibit the ability of cells to fix nitrogen [102, 103]. The binding to and reactions of these substrates with nitrogenase has allowed for the study of the enzyme’s biochemistry.

**Uptake hydrogenase**

The historical study of nitrogen fixation-related enzymes also includes study of the associated hydrogen-oxidizing uptake hydrogenase system in *Azotobacter*. This system consumes hydrogen gas, either nitrogenase-generated or exogenous [104, 105]. In the presence of a strong reducer at low pH, it can reduce protons to produce hydrogen [104–106], but these conditions are not found in vivo. The system is membrane-bound [105–107] and contains two peptide subunits, the large and small α and β subunits [105, 108]. These are encoded by the *hoxG* and *hoxK* genes, respectively [109], although the product of *hoxG* must be processed to remove a carboxyl-terminal stretch before it is functional [108, 110]. The enzyme contains a cofactor cluster containing iron and nickel, making it an NiFe hydrogenase [105, 110, 111]. Other enzymes encoded by the *hox* and *hyp* operons are necessary for activity but not directly involved. HypE is involved in processing the α subunit and insertion into the membrane [107]; HypB functions in nickel processing and insertion into the enzyme [111]; and HoxZ carries the hydrogen-derived electrons to the electron transport chain of the cells [109, 112, 113]. The functions of the other gene products are not as well defined, but they are also important [111, 112]. Electrons from hydrogen travel to HoxZ and then through the electron transport chain, reducing cytochromes d and b, but especially cytochrome c [114], and from there go on to reduce oxygen to water [113]. As *A. vinelandii*’s exposure to hydrogen mostly comes from nitrogenase, the activity of hydrogenase is closely linked to nitrogen fixation [111, 115]. This system will be explored in more detail below.

**Alternative nitrogenases**

The presence of alternative nitrogenase isoenzymes was hinted at in studies in the 1960s and early 1970s, by observation of the substitution of V for Mo [116, 117]. In 1980, Bishop and colleagues observed nitrogen-fixing pseudorevertants derived from nitrogenase-negative mutants [118], which were able to grow despite the presence of tungsten [4], an element that poisons the Mo nitrogenase [4, 119–121]. None of these pseudorevertants had a functional Mo nitrogenase [4], suggesting the presence of an independent enzyme system. Later, they observed an alternative DNR directly, expressed in the absence of Mo, using two-dimensional gel electrophoresis [25], and nitrogen fixation in strains with the Mo nitrogenase genes deleted [5]. The V nitrogenase was isolated soon after [35], followed by confirmation and isolation of a third nitrogenase containing neither Mo nor V but only Fe [26, 36, 37]. These systems were then studied more extensively in *A. vinelandii*.

**Nitrogen regulation**

The regulation of these different versions, by fixed nitrogen and by the presence or absence of the metals required by different versions, has been studied as well. The presence of Mo represses activity of the V and Fe nitrogenases while activating Mo nitrogenase activity [25, 38, 119]. Similarly, if Mo is absent, the presence of V represses the Fe nitrogenase [37, 38, 41, 119]. If neither is present or if the culture exhausts their supply, *A. vinelandii* depends on the Fe nitrogenase to grow [37, 40].

Regarding fixed nitrogen sources, *A. vinelandii* is capable of using inorganic sources (ammonium, nitrate, nitrite) and several organic sources: aspartate, asparagine, glutamate, adenine and urea [122]. Ammonium salts are the most potent in repressing all forms of nitrogenase activity [122–125], at concentrations of 25 µM and above [126]. The mechanism of this shut-off, which is almost immediate, seems to be a decrease in proton motive force, restricting the access of nitrogenase to reducing equivalents that it requires [124]. Factors that modify the force of this repression include the amount of oxygen and the cells’ potential respiratory rate (too little oxygen, or too much without an adequate increase in respiration, enhances the repression of nitrogenase) [6, 127]; high pH also enhances repression, which provides supporting evidence for a mechanism involving proton motive force [127]. Other fixed-nitrogen compounds can induce repression in *A. vinelandii* also: nitrate salts, especially when cells are pre-adapted to them [122, 128], and urea [129] can fully repress nitrogen fixation; and organic compounds such as aspartate, adenine, yeast extract or casamino acids can repress nitrogenase partially [122, 125, 130]. *A. vinelandii* does not appear to store or ‘park’ nitrogenase protein inactivated by fixed nitrogen-induced regulation over long periods [6, 84, 88], in the way that organisms such as *Rhodospirillum rubrum* store nitrogenase via ADP-ribosylation [131]; inactive protein is broken down in *A. vinelandii*.

**Nitrogen fixation under oxygen**

As an obligate aerobe [71, 132], *A. vinelandii* has also been a useful model for the study of aerobic nitrogen fixation and how bacteria protect their oxygen-sensitive enzymes from oxidative damage: through enhanced respiration (‘respiratory protection’), reversible conformational inactivation via...
the FeSII/Shethna protein and other methods (such as superoxide dismutase). All DNR versions can be irreversibly inactivated by oxygen and its reactive species, preventing *A. vinelandii* from growing diazotrophically [23, 72, 73, 84, 133]. So these bacteria use various strategies to protect their enzymes from oxygen. Like most aerobes, *A. vinelandii* produces superoxide dismutase, SodB; this production is proportional to oxygen exposure, especially when fixing nitrogen [134]; catalase is also produced, but not proportional to oxygen exposure [134]. *A. vinelandii* also produces catecholate siderophores that can chelate iron and prevent Fenton chemistry and the generation of reactive oxygen species; this production is also proportional to oxygen exposure [134]. So these bacteria use various strategies to protect their enzymes from oxygen. Like most aerobes, *A. vinelandii* produces superoxide dismutase, SodB; this production is proportional to oxygen exposure, especially when fixing nitrogen [134]; catalase is also produced, but not proportional to oxygen exposure [134].

However, the most important strategy in *A. vinelandii*’s arsenal is respiratory protection, in which cells ‘waste’ oxygen by adapting their respiratory rate to the level of oxygen exposure [7, 9, 72, 101, 140–142]. Electrons flow from NADH dehydrogenase II down the cytochrome *bd* branch of the electron transport chain [143, 144]; this branch is mostly decoupled from phosphorylation [23, 72, 145–147] and produces very little superoxide [143]. This results in increased production of carbon dioxide [7, 142] and higher rates of respiration [23, 72, 141] due to augmented aldolase activity, which directs carbon through the pentose phosphate cycle [142]. This affects yields and specific growth rate, because the bacteria devote energy and carbon to respiratory protection rather than other metabolic pathways [9, 132, 141, 142, 148]. However, rather than creating an intracellular anoxic environment for enzyme protection, as has been supposed, there is evidence that respiratory protection instead allows enzymes to remain in more oxygen-tolerant reduced states [84, 133, 149]. A recent study explores in detail the costs associated with this respiratory protection [150].

**Respiration**

*A. vinelandii* has also been a model for the study of respiration in general, including aspects of the tricarboxylic acid cycle, the electron transport chain and its branches, and the substrates involved in respiration. *A. vinelandii*’s electron transport chain is interesting because it comprises multiple branches with different terminal oxidases in each [143, 146, 147, 151], a setup that is essential for its adaptation to varying levels of oxygen. NADH and L-malate are the physiological substrates that donate electrons to begin the chain [147, 152]; NADPH may be used indirectly by passing electrons to NADH through a transhydrogenase [153]. From there, all electrons go through ubiquinone (Qₐ) before passing to the different branches [143, 147].

There are two main branches after ubiquinone. One is composed of *b* and *c* cytochromes and ends in a haem-copper oxidase; this branch has lower activity but higher affinity for oxygen, and has high phosphorylation activity, and is especially important in low-oxygen conditions [23, 143, 151, 154]. Some studies suggest that this branch may be further split into multiple branches with similar properties [151, 154, 155]. The other main branch consists of cytochrome *bd* oxidase, a quinol oxidase that is faster but has relatively lower affinity for oxygen, and has almost no ATP generation ability [23, 144, 156, 157], although other studies have found that it is not completely uncoupled from oxidative phosphorylation [158, 159]. *A. vinelandii* up- or downregulates these branches depending on the redox conditions in its environment: the *bd* branch is important in highly oxic conditions, and the other at lower oxygen levels [143, 144, 154, 156, 157]. This organism’s setup represents an important strategy of adaptation to a complex redox habitat.

**Agriculture**

*A. vinelandii*’s roles in soils and agriculture have also been examined and even commercialized [160]. Considering the problems that plague industrial nitrogen fertilizer use – depleted soil quality, environmental pollution, intensive energy use [161–163] – biological nitrogen fixation in soils is of significant interest [164]. *Azotobacter* is known to dwell in soils worldwide [1, 165, 166], although its abundance depends on the specific soil conditions [167]. Cysts of *A. vinelandii* can survive at least 10 years in dry soil [166], and the vegetative cells can grow quite well on the phenolic acids and other nutrients in soil [168]. *A. vinelandii* is also a plant growth-promoting rhizobacterium, living near plant roots and potentially increasing crop yields [169, 170]. The most obvious effect is *A. vinelandii*’s production of fixed nitrogen compounds. Plants are capable, at least under certain conditions, of utilizing fixed nitrogen of bacterial origins [171–173]. *A. vinelandii* itself can be modified not just to fix nitrogen to ammonium but to excrete excess into its surroundings, acting directly as a fertilizer [17, 174–177], producing concentrations in medium as high as 35 mM [176]. Patent applications have been filed for this technology [178, 179]. However, *A. vinelandii* can also increase plant growth by other methods, such as increasing the nodulation activity of local rhizobia around legume roots [180] and by producing plant growth-promoting hormones such as indole acetic acid (IAA) [181–183]. Hydrogen produced from nitrogen fixation can boost growth of hydrogen-oxidizing microbes beneficial to plants, possibly including *A. vinelandii* itself [184]. Some strains show potential for plant pathogen biocontrol via antifungal activity [185]. Finally, *A. vinelandii* can enhance the nutritional value of crops, by increasing their vitamin content [186] and protein quality and quantity [183].

Through these historical areas of research, *A. vinelandii* has contributed much valuable knowledge. Fig. 2 provides an...
overview of the organism’s fundamental metabolism. Moving forward, using modern systems and synthetic biology techniques and approaches, study of this organism has great potential to contribute to multiple valuable areas of research.

PROMISING AREAS FOR A. VINELANDII RESEARCH

A. vinelandii has also been studied for its various biotechnological potentials. These include the production of hydrogen from the nitrogenases, natural polymers of industrial interest and application of nitrogenase to alternative substrates.

Polyhydroxyalkanoates (PHAs)

A. vinelandii produces hydrocarbon polymers in certain conditions. This was discovered as early as 1937 by the observation of lipid-like granules in Azotobacter cells [33]; these granules were later identified as PHB [187], and confirmed as present in A. vinelandii in 1958 [188]. The amount of PHB in A. vinelandii cells peaks during the encystment process and then falls to a lower level as the cyst matures [189]. These polymers have certain advantages over petroleum-derived polymers: they are a form of renewable bioplastic [190–192]; they are biodegradable [193, 194]; and they can make biocompatible medical devices [195–199] or scaffolds for cell cultures [200]. However, compared to petroleum thermoplastics, bacterial PHAs have less versatility and are not always cost-competitive, so more research and development is needed to make them viable products.

A. vinelandii primarily produces PHB. In a standard batch culture, most strains start producing PHB in late exponential phase, and then consume the polymer in stationary phase [50, 201, 202]. PHB can accumulate to up to 70% of the cells’ dry weight [24, 49, 50]. As mentioned, there is a connection between PHB production and encystment; PHB may be a storage polymer for this process in strains that produce cysts [202]. In more controlled conditions, oxygen limitation induces its production in Azotobacter species [24, 148, 201, 203]. The cells then reassimilate the PHB if the oxygen limitation is lifted [24, 203]. Oxygen limitation can cause buildup of excess reductant, which PHB formation can alleviate by acting as an electron sink [203, 204]; impairment of NADH oxidation causes a similar effect [50, 205]. Iron limitation also may induce PHB production [201]. The carbon substrate is also important: acetate and ethanol allow for little or no PHB production, compared with that produced from sugars or butanol [49, 50, 202].

As PHB production and degradation is an important metabolic pathway in Azotobacter, it is subject to regulation by a number of different factors. Most directly, the PhbR protein upregulates the PHB-producing operon (phbBAC) to which its gene (phbR) is adjacent [206]. The stationary phase-associated sigma factor (σ38 or RpoS) also plays a role in upregulating PHB production [206, 207], and the global two-component response regulator system, GacS/GacA, is also involved in this upregulation [208–210]. Other regulatory factors are involved as well. The arrF gene encodes a small regulatory RNA, ArrF, expressed in conditions of iron limitation, which regulates the expression of iron-containing proteins such as superoxide dismutase and the FeSII/Shethna protein [211]. Knocking out the arrF gene increases the production of PHB around 300-fold, even in well-aerated conditions; the ArrF small RNA (sRNA) represses genes related to PHB production [201]. Other sRNAs, regulated by the GacS/GacA system, include RsmA, which represses PHB production [209]. CydR (Fnr) is a transcriptional regulator protein that controls the low-affinity cytochrome bd branch of the electron transport chain; knocking out the cydR gene results in overproduction of PHB-producing proteins [212]. The rhodanese protein RhdA, which may regulate protection of A. vinelandii from oxidants, represses PHB production [213]. PtsP, involved in regulating nitrogen fixation and respiratory protection in carbon-limited conditions, enhances the production of PHB [214]. It is likely that this summary is not an exhaustive list of all relevant factors, as some may remain to be discovered, but it is clear that polymer production is important to different aspects of A. vinelandii metabolism.

For strains of A. vinelandii that produce it, the production and regulation of alginate is also related to PHB metabolism in interesting ways. Alginate is a slimy polysaccharide of varying composition that many strains of A. vinelandii produce, while other, ‘non-gummy’, strains do not [43]. In some conditions, such as low aeration, strains that overproduce PHB also produce more alginate than wild-type strains [205]. The GacS/GacA system upregulates the production of both polymers [208, 210], and in some conditions, mutations of both PHB- or alginate-related genes reduce the production of alginate [215]. PHB and alginate are both involved in cyst structure and encystment and germination processes of A. vinelandii [202, 216–218], although strains unable to produce PHB can still encyst [219]. However, PHB and alginate production may compete for the same carbon substrate, so in some conditions, knocking out the ability of A. vinelandii to produce PHB or alginate increases yields of the other several-fold [220, 221].

To solve the problems of cost-competitiveness and versatility of properties, a number of approaches have been attempted. For example, William Page and Olga Knop developed a non-gummy strain of A. vinelandii, strain UWD, that overproduces PHB constitutively; they transformed non-gummy strain UW (also known as (aka) CA or OP [43]) with genetic material from a gummy PHB-overproducing strain [50]. Strain UW consumes glucose more quickly than its parent (strain UW), and unlike UW in which low aeration induces PHB production, higher aeration correlates with higher PHB production in UWD (to a point) [50, 51]. NADH oxidation is impaired in strain UWD, so cells use PHB production as an electron sink to prevent reducing equivalents from building up [50, 205]. UWD produces yields of PHB almost sevenfold higher than its parent [50]. Use and development of UWD and other over-producing strains (which could be engineered by
modifying the regulatory factors mentioned previously [222]) could improve the efficiency and competitiveness of bioplastic production.

William Page's group and others have also studied the use of low-cost sources of carbon as substrates to replace costly, purified sugars and other compounds. For example, corn syrup, cane or beet molasses, and malt extract allow PHB production comparable to glucose [28, 49, 51, 52], and beet molasses actually stimulates the growth of *A. vinelandii* UWD more than other substrates [49]. One study estimated that, in 1992 at least, PHA production from UWD using beet molasses could be less expensive than polypropylene production [28]. As a low-cost source of nitrogen, researchers tested fish peptone with strain UWD [223, 224]; added nitrogen greatly increases the yield of PHB [50, 51, 223], and adding fish peptone more than doubles the PHB yield from molasses [223]. A two-stage process, with beet molasses in the first stage for biomass/protein production and then fish peptone and raw sugar in the second stage for PHB, increases production even further [224]. Peptone also increases pleomorphism in *A. vinelandii* by weakening the structural integrity of the cell wall, resulting in weirdly shaped 'fungoid' cells [34, 223]; this actually proves advantageous, as it facilitates extraction of the polymer from cells [223]. Other low-cost substrates, such as swine wastewater, olive mill wastewater and cheese whey, also show promise for production of PHAs [225–229].

To address the problem of versatility in the properties of PHA bioplastics, some have studied the possibility of producing copolymers in *A. vinelandii*, instead of just PHB. When valerate (a five-carbon fatty acid) is added to the growth medium, *A. vinelandii* strain UWD produces poly(hydroxybutyrate-co-hydroxyvalerate) or (P(HB-co-HV), a copolymer with two kinds of monomer and with improved material properties compared with PHB [28, 230]. PHA yield decreases under these conditions, however [230].

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**Fig. 2.** Overview of *A. vinelandii*'s metabolic pathways, showing inputs, outputs, pathways, and regulation for carbon, nitrogen and electrons. Top: low-oxygen conditions. Bottom: high-oxygen conditions.
use of swine wastewater permits copolymer formation without added valerate, but supplementation improves yields [226, 229]. The development of other polymers and processing methods could increase the penetration of PHAs into the plastics market.

By creative use of what is known regarding PHA regulation, combined with improved design of production processes, *A. vinelandii* could act as a catalyst for competitive, efficient biodegradable biopolymer production.

**Alginates**

Alginates are slimy polysaccharide polymers mainly consisting of two sugar acids, (1-4)-β-D-mannuronic acid and α-L-guluronic acid. Bacterial species in the family Pseudomonadaceae, including those in the genera *Azotobacter*, *Pseudomonas* and *Azomonas*, produce this polymer [231], as do brown algae. *A. vinelandii* converts hexoses such as glucose to trioses through the Entner–Doudoroff pathway, and then back to alginate via gluconeogenesis [232].

In *A. vinelandii*, depending on the conditions, certain strains produce alginate and others do not. Wild-type strains produce it, but ‘non-gummy’ strains such as strain OP (aka UW or CA) can be isolated that generally do not [43]. Sequencing the genome of strain DJ, an offspring of strain CA, revealed a transposon insertion into the *algU* gene, which encodes an activator of alginate production, which explains its non-gummy phenotype [11]. However, PHB-overproducing strain UWD also produces much more alginate than its non-gummy parent (strain UW) when oxygen-limited in stationary phase [205], so environmental conditions and regulatory factors other than AlgU also influence production of the polymer.

Alginate in *A. vinelandii* is associated (and, in fact, required) for encystment [217, 233]; non-gummy strains do not form cysts [217, 234], although they may form cyst-like structures [234]. Alginate forms a protective part of the cyst coat, helping it to resist desiccation [189, 218]. Its rate of production and molecular properties (molecular weight, ratio of mannuronic to guluronic acid, degree of acetylation) vary depending on the level of oxygen in the cells’ environment, possibly indicating a role for alginate in protecting the cells from excess oxygen [136]. Non-gummy strains also show increased susceptibility to reactive oxygen species [235]. The presence of alginate also affects the natural competence and transformation efficiency of *A. vinelandii*; gummy strains are less easily transformed [29, 53], although protocol modifications can enhance their competence [29, 53].

As with PHB, several independent factors regulate the production of alginate in *A. vinelandii* via transcriptional regulation of the *alg* operon starting with *algD*, which encodes GDP-mannose dehydrogenase, the rate-limiting enzyme in the process that converts GDP-mannose to GDP-mannuronic acid. The AlgU protein (aka RpoE), a putative sigma factor, upregulates the *alg* operon [235]; when *algU* is knocked out, such as in strain OP (aka UW or CA) [11, 235], the cells usually do not produce alginate [43, 235].

Strain UWD, a derivative of strain OP, does produce alginate when oxygen-limited, however; in this case, *algD* may be upregulated by RpoD (aka σ70) [205]. The products of the mucABCD and other muc genes are negative regulators of the *alg* operon [235–237]. Additionally, the GacS/GacA global two-component response regulator system and the stationary phase-associated sigma factor (RpoS) activate alginate production [207, 208], the former via the PTPN global regulatory system and sRNAs that inhibit the repressor protein RsmA [209, 210].

Alginates are products of interest industrially; they can be used as stabilizing, gel-forming or thickening agents in various processes in the food, pharmaceutical and cosmetics industries [238, 239]. Alginates from *A. vinelandii* perform better in wound dressing applications than those of marine origin [240]. Alginic beads can also be used to store beneficial bacteria and inoculate plants by planting the beads along with plant seeds [241]; plant growth-promoting gummy strains of *A. vinelandii* could actually be packaged directly into beads using the alginate they produce. Various approaches are being taken to develop processes in *A. vinelandii* to produce higher amounts and diverse forms of alginate for various potential uses [242]. For example, regulating the oxygen transfer rate [243–245] and oscillation of dissolved oxygen [246] can affect the expression of alginate-related genes [247] and the average molecular mass of the final alginate product, and to a lesser extent its yield [244] and degree of acetylation (another important property) [245]. To regulate this, some have compared shake flask to bioreactor cultures and the power each consumes [248], as well as two-stage reactor processes with high aeration followed by low aeration [221]. Optimizing the exposure of cells to CO₂ increases alginate yield and molecular mass [249], and adding MOPS to the culture medium can increase the degree of acetylation [250]. Finally, genetic engineering can improve alginate production even further: knocking out PHB production increases alginate yield several-fold [220, 221], although at the cost of some molecular mass [221], and reducing the amount of ubiquinone in cells also increases alginate yield [251]. A double-knockout of *phbR* and *muc26* produces less alginate but at a very high molecular mass [215], and knocking out alginate lyase may increase mass even further [252]. Controlling and engineering *A. vinelandii*’s family of mannuronan C5-epimerases could improve production of specific alginate products with better mechanical properties [253]. Optimizing and combining these strategies with process engineering could result in a competitive system for production of alginates from *A. vinelandii*.

**Other interests**

Other avenues of research include using *A. vinelandii* to detoxify cyanide in wastewater from cassava processing [15]. *A. vinelandii*’s nitrogenases accept cyanide as a substrate, converting it to ammonia and methane [254–256], or larger products such as methylamine in the case of the V nitrogenase [256]. When incubated with wastewater
containing 4 mM cyanide, *A. vinelandii* reduced the concentration by almost 70% within 66 h. Additionally, the ammonia and methane produced from this may be harvested for fertilizer and energy. Other kinds of wastewater can be treated using *A. vinelandii* as well, to remove organic carbon without the addition of fixed nitrogen [257] or to remove potentially toxic polyphenol compounds [258].

Another potential use of *A. vinelandii* is providing fixed nitrogen for other industrially relevant microbes. Some algae and other microbes are capable of taking up bacterial siderophores and assimilating their metal contents [259, 260], but siderophores also contain fixed nitrogen, so those produced by *A. vinelandii* may act as a nitrogen source for other species. For example, algal species of industrial interest, such as *Neochloris oleoabundans* and *Scenedesmus* sp. BA032, are capable of growing with purified *A. vinelandii* siderophores as the sole nitrogen source, or even in coculture with the bacteria themselves in the case of *Scenedesmus* [16]. The algae in that study preferred azotobactin rather than the catechol siderophores, but other algal species may have different capabilities [16]. Algae could also take up excreted ammonium directly from modified strains of *A. vinelandii* [17, 174, 261]. One fascinating application of this is pairing ammonium-exporting *A. vinelandii* with a carbohydrate-exporting phototroph to create a self-sustaining co-culture for the production of PHB [262]; this coculture can also be embedded in a hydrogel matrix for increased efficiency [263].

A modified version of *A. vinelandii*’s alginate lyase enzyme, AlgL, could be used in combination with antibiotics to treat infections of biofilm-forming pathogens [264], and *A. vinelandii* can also be used to produce other valuable enzymes, such as L-asparaginase [265].

Hydrogen

*A. vinelandii* is capable of consuming hydrogen gas and generating energy from it, using its membrane-bound uptake hydrogenase enzyme complex. Unlike other microbial hydrogenases that act reversibly and produce H₂ in many conditions [266], *A. vinelandii*’s hydrogenase is unidirectional in its H₂ oxidation except when exposed to low pH and a strong reducing agent [104, 105].

*A. vinelandii*’s hydrogen consumption could be useful in various ways. The energy hydrogen provides may contribute to nitrogen-fixing activity [267], ATP production [145, 268] and mixotrophic growth with mannose [269]. It may also help protect nitrogenase from inactivation by oxygen, via respiratory protection [267, 268]. It could supply some extra energy to the processes of interest mentioned before, or for others. For example, in a similar species, *Cupriavidus nectarius* (formerly *Ralstonia eutropha*), Torella *et al.* developed a system in which photovoltaic cells split water to produce hydrogen, which bacteria then consumed to generate isopropanol, effectively converting solar power to biofuel [270].

As mentioned previously, in addition to consuming molecular hydrogen, *A. vinelandii* is also capable of producing it, as a byproduct of the nitrogen fixation reaction [26, 39, 271]. Even under a high-pressure nitrogen atmosphere, nitrogenase evolves at least one molecule of hydrogen per molecule of nitrogen fixed [91]. Although in wild-type *A. vinelandii* the uptake hydrogenase consumes almost all the hydrogen produced [268, 272], hydrogen production can be observed when the hydrogenase is not functioning, allowing *A. vinelandii* to be studied as a model of aerobic, heterotrophic hydrogen production [18].

This can be accomplished in a number of ways. The bacteria’s natural competence, discussed previously, can be exploited to delete or disrupt essential hydrogenase genes [107, 111–113, 272]. Spontaneous mutants can also be isolated by mutagenesis or selection [4, 18, 273]. Chemical methods can also be used to inactivate the hydrogenase, temporarily or irreversibly. Carbon monoxide and cyanide can inactivate the enzyme [274, 275], as can nitric oxide [106] and even acetylene in sufficiently high concentrations [267, 268]. Prevention of active hydrogenase synthesis is also possible by addition of chelators such as EDTA or NTA, which bind nickel and iron and prevent their incorporation into hydrogenase active centres [110, 276, 277].

The metal tungsten (W) also can affect hydrogen production. It is chemically similar to molybdenum, and can be incorporated into the Mo nitrogenase as an iron–tungsten cofactor (FeWco) in place of the normal FeMoco [120, 121]. This appears to inhibit all functions of the enzyme, poisoning it [120, 121] and stopping the growth of wild-type *A. vinelandii* [4, 119, 120]. The extent of this inhibition can be affected by the concentration of molybdenum present [120]. Strains of *A. vinelandii* that do not repress alternative nitrogenases can grow in the presence of tungsten, by relying on these alternatives for nitrogen fixation [4, 119].

Other compounds can affect the flux of electrons through nitrogenase as well. By removing substrates external to the nitrogenase system, such as in a nitrogen-free atmosphere of argon, the electron flux can be directly entirely to proton reduction. This results in a transient production of 1.2- to 11-fold more hydrogen than when nitrogen is present [26, 39, 271], depending on substrate, specific atmospheric composition and nitrogenase isoenzyme.

Modifying hydrogen production by making point mutations to nitrogenase enzymes is also possible. By changing NifD position 191 from glutamine to lysine, most electron flux is redirected toward hydrogen instead of other substrates, but the total flux may be reduced [278–280], although this reduction may reflect only a lower proportion of active protein [279]. Modifying NifD position 195 from histidine to glutamine also redirects electrons from acetylene to protons [279]. Recent work has suggested that hybrid systems combining proteins from different organisms could possess more varied and specific catalytic capabilities [281].
In summary, \textit{A. vinelandii}'s hydrogen production parameters hinge on the interplay between hydrogenase and nitrogenase enzymes. Inactivating or removing the uptake hydrogenase allows nitrogenase-derived hydrogen to escape and be measured or collected. The amount of hydrogen produced then depends on a number of other factors: nitrogenase version, oxygen exposure, metabolic regulation and other attributes. Optimizing the system could allow \textit{A. vinelandii} to serve as a valuable catalyst, or at least a model of a novel hydrogen-producing organism.

CONCLUSIONS

Study of \textit{A. vinelandii} and its cousin species has contributed much to bacteriological knowledge for the past century, and they continue to represent important species with great potential for research and industrial interest.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References

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15. Kokusun S, Vichithphan S, Laopaiboon L, Vichithphan K, Han J. Growth and cyanide degradation of \textit{Azotobacter vinelandii} in cya-

16. Villa JA, Ray EE, Barney BM. \textit{Azotobacter vinelandii} siderophore can provide nitrogen to support the culture of the green algae \textit{Neochloris oleoabundans} and \textit{Scenedesmus} sp. BA032. \textit{FEMS Microbiol Lett} 2014;351:70–77.

17. Barney BM, Eberhart LJ, Ohlert JM, Knutson CM, Plunkett MH. Gene deletions resulting in increased nitrogen release by \textit{Azoto-


22. Setubal JC, Almeida NF. The \textit{Azotobacter vinelandii} genome: an update. In: Bruijn FJ (editor). \textit{Biological Nitrogen Fixation}. Hobo-


26. Chisnell JR, Premakumar R, Bishop PE. Purification of a second alternative nitrogenase from a \textit{nisHDK} deletion strain of \textit{Azoto-

27. Wyss O, Neumann MG, Socolofsky MD. Development and germi-


32. Herter S, Schmidt M, Thompson ML, Mikolasch A, Schauer F. A new phenol oxidase produced during melanogenesis and encystment stage in the nitrogen-fixing soil bacterium \textit{Azotobac-


179. das HK, Bageshwar UK, Srivastava M. Recombinant nitrogen fixing microorganism and uses thereof. 2016. Available at: http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&i=
1&vid=G&i50&PTXTRK1(I=nifLCLTX.or+nifLDCTX)&OS=ACLM
nifL&RS=ACLM/nifL. [Accessed August 24, 2016].


1&vid=G&i50&PTXTRK1(I=nifLCLTX.or+nifLDCTX)&OS=ACLM
nifL&RS=ACLM/nifL. [Accessed August 24, 2016].


212. Romo-Uribe A, Meneses-Acosta A, Domínguez-Díaz M.
213. Saito T, Tomita K, Juni K, Ooba K.
215. Stevenson LH, Socolofsky MD.
216. Jackson FA, Dawes EA.
217. Senior PJ, Dawes EA.
218. Page WJ, Tindle A, Chandra M, Kwon E.
220. Castañeda M, Sánchez J, Moreno S, Núñez C, Espín G.
221. Castañeda M, Guzmán J, Moreno S, Espín G.
222. Hernández-Eligio A, Moreno S, Castellanos M, Castañeda M,
Núñez C et al.
223. Trojo A, Moreno S, Cocotti-Yañez M, Espín G.
224. Jung YS, Kwon YM.
227. Segura D, Espín G.
229. Sillman CE, Casida LE.
230. Sillman CE, Casida LE.


272. Linkerhänger K, Oelze J. Hydrogenase does not confer significant benefits to *Azotobacter vinelandii* growing diazotrophically


