Establishment of artificial symbiosis between *Lemna minor* and the diazotrophic bacterium *Azotobacter vinelandii*, and elucidation of the mechanisms of bacterial plant growth promotion

（ウキクサ *Lemna minor* と窒素固定細菌 *Azotobacter vinelandii* の人工共生系構築と植物成長促進機構の解析）

Doctoral dissertation

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1. General introduction

1.1. Introduction to Duckweed

Duckweed, family *Lemnaceae* (Acosta et al., 2021); is a small aquatic monocotyledon that grows on or just beneath the surface of still or slow-moving bodies of fresh water and wetlands. Having a simple structure that lacks an obvious stem or leaves, it's composed of one or few leaf-like structure called "fronds" often with air pockets (aerenchyma) that allow it to float on or just under the water surface, and none or a few root or rootlet depending on the species (Richards & Sculthorpe, 1968). The size of the fronds also varies depending on the species (1.0- 8.0 mm) which is smaller compared to other aquatic macrophytes (Lämmler et al., 2014). The fronds of duckweed grow either singly or connected in small groups or networks multiplying by vegetative reproduction and form a thick blanket of biomass on water surface (Correll & Correll, 2011; L. L. Rusoff et al., 1980) with an outstanding doubling time of maximally 1.4 days (Frick, 1985). Thus duckweed family has been dubbed as "Darwinian Demons" due to their relentless reproductive capacity (Kutschera & Niklas, 2015). Due to global distribution, extreme reduction of morphological characteristics and high phenotypic plasticity to environmental conditions, the taxonomy and the phylogeny of the family *Lemnaceae* still has an ongoing dispute (Bog et al., 2010; Les et al., 2002). About 40 species of duckweeds are distributed among the following five genera which are ubiquitous almost all over the globe: *Lemna*, *Landoltia*, *Spirodela*, *Wolffiella* and *Wolffia*. While each of the genera have their own unique features, the *Landoltia* and *Lemna* species are often utilized in phytoremediation in waste water treatment and consequent biomass production (Cheng & Stomp, 2009) and specially *Lemna minor* has been explored for studies on their host microbe interaction (Ishizawa et al., 2017b, Yamakawa et al., 2018).

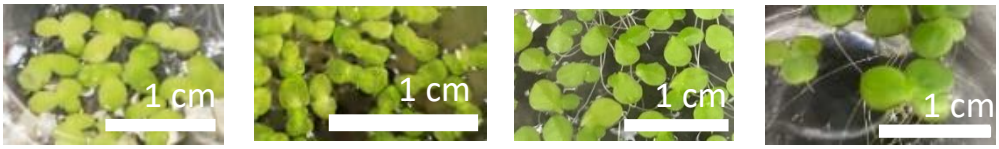


Figure. 1. Different species in family Lemnaceae. From left to right, *Lemna minor, Wolffiella hyalina, L. gibba, Spirodela polyrhiza*

1.1.1. Applications of Duckweed

Duckweed *L. minor* has been used in aquaculture, livestock production, poultry, pharmaceuticals, biofuels, toxicity testing, and environmental monitoring and for wastewater treatment for decades. Following are different applications of duckweed.

1.1.1.1. Animal feed

The high quality and promotional effect of duckweed protein on rat growth (Dewanji & Matai, 1996) poultry production, duckling growth (Hamid et al., 1993), ruminant growth (L. Rusoff et al., 1978) carp, tilapia fish growth (Dyke & Sutton, 1977; Hassan & Edwards, 1992; R. A. Leng et al., 1995; Skillicorn et al., 1993); has been reported. Duckweed cultivated at fast-growing conditions with high protein contents has the potential to substitute fish meal or soybean meal which are typical animal feed. Duckweed powder when used as supplement rather than sole nutrient source has a higher potential of supplying essential amino acids for egg production. Duckweed biomass grown in optimum growth condition has already been used as a sole supplier of nutrient for many animals (Culley et al., 1973) as well as supplemental human food (Kibbutz, 1991) due to its high protein content (Appenroth et al., 2017). Protein production and starch accumulation in duckweed is interchangeable depending on the growth condition. When grown under stressed condition, the duckweed decreases protein production level and in turn accumulates high amount of starch, which then can be applied as feedstock for biofuel production (Su et al., 2014).

1.1.1.2. Human food

Eaten in Thailand, Laos and Cambodia for ages, duckweed has been reported to possess proteins as per FAO recommendations (L. L. Rusoff et al., 1980). Recently, duckweed especially *W. hyalina* and *W. microscopica* (Appenroth et al., 2017) is being considered as human food or food supplements. Duckweed has been tested to be free of cytotoxicity and anti-proliferative effect on human cell lines (Sree et al., 2019). The passive interest for making duckweed a human food is turning into an active research area (de Beukelaar et al., 2019). However there are some legal issues to be taken care of before duckweed can be marketed as human food (van der Spiegel et al., 2013).

1.1.1.3. Plant factory

Duckweed has been suggested as a suitable protein expression system for production of recombinant proteins (Stomp, 2005; Yamamoto et al., 2001). Genetic manipulation has been introduced to *L. minor* for production of pure and high form of monoclonal antibodies (Cantó-Pastor et al., 2015; Cox et al., 2006; Naik et al., 2012). Moreover, duckweed is edible and thus offers an attractive system for oral vaccines (Popov et al., 2006; Rival et al., 2008).

1.1.1.4. Production of Biofuel

Duckweed *S. polyrhiza* grown in diluted animal effluent can be manipulated to yield high amount of starch. The readily hydrolysable and fermentable biomass produced 50% higher ethanol than that of maize-based ethanol production (J. Xu et al., 2011; Cui & Cheng, 2015).

1.1.1.5. Biomonitoring

Duckweed has been applied for eco-toxicological studies and environmental monitoring of pollutants (Mufarrege et al., 2010; Senavirathna et al., 2014; Sinha et al., 2005.; Tkalec et al., 2005; Tkalec et al., 2007). Using duckweed experiments to develop national and international standards for toxicity studies has been proved to be more efficient than animals (Bog et al., 2010; Brain et al., 2012; Brain & Solomon, 2007; Hughes et al., 2009). Duckweed-microbe bioassay for

detecting herbicide linuron degradation has been developed using *L. minor* (Hulsen et al., 2002).

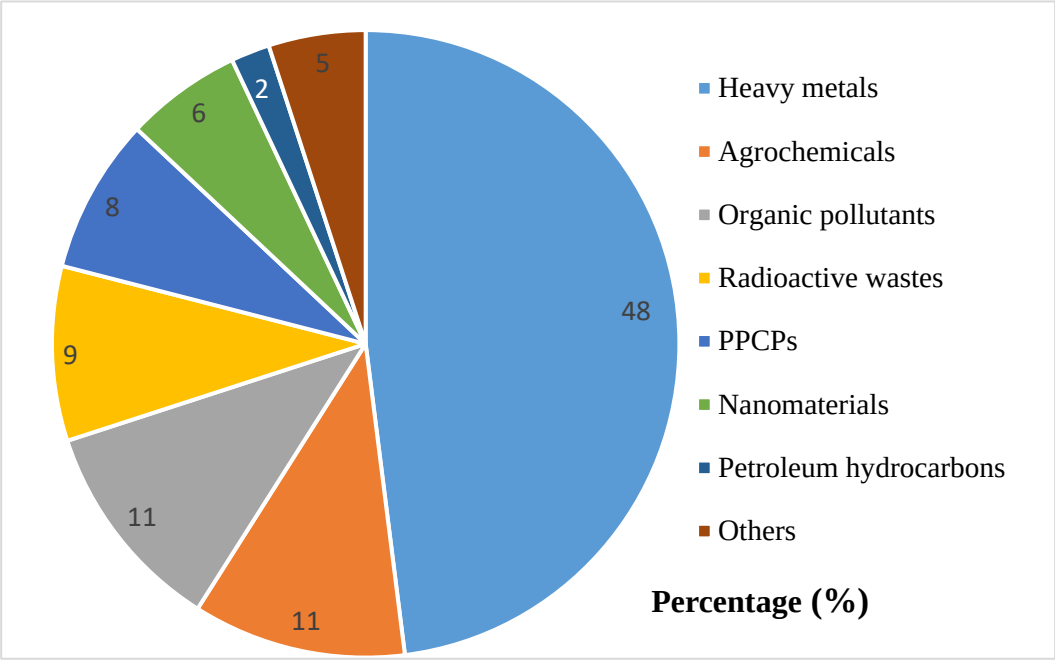


Figure 2. Categories of pollutants remediated by *L. minor* adopted from (Ekperusi et al., 2019)

1.1.1.6. Duckweed phytoremediation

Increased industrialization from 20th century towards the 21st has made the environment laden with increasing levels of toxic effluents that are released into the ecosystem. Duckweed *L. minor* has been intensively applied to absorb or degrade the chemical pollutants from the environment (Figure 2) (Mohedano et al., 2012). BOD (Biological oxygen demand) and phosphate concentration reducing capability of duckweed in municipal effluents is greater than conventional primary and secondary treatment (Priya et al., 2012). Thus, duckweed has been applied for wastewater treatment (Bonomo et al., 1997; Chaudhary & Sharma, 2007). Duckweed subsequently produces useful biomass (J. Xu & Shen, 2011) depending on the effluent type. Combination of two or more

types of duckweed has been reported to have a greater effect on organic pollutant removal (Van Echelpoel et al., 2016; Yilmaz & Akbulut, 2011; Zhao et al., n.d.). Phytoremediation effect of duckweed in urban wastewater is also notable when it is used in a combination of macrophytes (Farid et al., 2014).

1.1.2. Current state of duckweed production and its challenges

There is a gap of knowledge in the mass production of duckweed *Wolffia globosa* as human food. *Wolffia globosa* is a member of family Lemnaceae with high protein and vitamin B12 content (Sela et al., 2020). Duckweed is one of the safest alternative protein source in the world in terms of allergens and phytotoxicity (van der Spiegel et al., 2013). However, mass production of duckweed in technical environment can create safety concerns (Markou et al., 2018). Moreover, uncertain types and amounts of microbial load renders it beyond the limit of human consumption. It is impractical to grow duckweed in a completely aseptic condition since many of the vitamin such as Vitamin B12 and metabolites may be provided to duckweed from the microbial side (Bakterien & Wasserlinsen, 2016). In an open air duckweed farming condition, addition of inorganic mineral nitrogen fertilizer risks increasing algal growth while use of complex organic compounds increases the bacterial load in the duckweed. Both of these extremities hamper duckweed quality and yield drastically (Khairina, 2021).

1.2. Plant growth promoting bacteria (PGPB) and their mechanisms

1.2.1. Rationale for artificial duckweed holobiont construction

In natural environment, duckweeds are colonized by numerous indigenous bacteria. Although it is an aquatic plant, bacterial communities are similar to a terrestrial plant (Acosta et al., 2020). The natural bacterial community is conserved and in some cases resilient to an extraneously added PGPB strain (Ishizawa et al., 2020). But for large scale production of duckweed, reliance on the indigenous bacterial community alone is not sufficient as individual members

of the community have shown both growth promotion and growth inhibition effects (Ishizawa et al., 2017a). This creates the need for construction of an artificial holobiont community with maximum PGP potential. Such a stable symbiosis has already been attempted where PGPB *Pseudomonas fulva* PS6 and *Acinetobacter calcoaceticus* P23 finds their own niches in root and fronds respectively while promoting the growth of *L. minor* (Yamakawa et al., 2018). The holobiont community may be composed of PGPB with certain properties such as nitrogen fixation, protection from predators and competitors, production of microenvironment modifier and/or growth regulator compounds.

1.2.2. PGPB in soil

Plant growth promoting bacteria are a group of bacteria or bacterial communities that positively affect the growth of a plant directly or indirectly by associating with a plant either by surface attachment or endophytic association. Application of these beneficial bacteria for the promotion of terrestrial plant growth has been utilized for over 60 years (Ahemad et al., 2014). In the terrestrial environment, plants have developed an intimate relationship with the bacteria which are crucial for their survival (Levy et al., 2018). These bacteria/bacterial communities provide the plant with myriad benefits like resistance to adverse environmental stresses such as water and nutrient deficiency, heavy metal contamination and plant pathogens and by production of plant hormones such auxin/IAA (Patten et al., 1996), ACC deaminase (Glick et al., 2014), cytokinin (Arkhipova et al., 2005), gibberellin (Taller et al., 1989), nitrogen fixation (Ahemad et al., 2014), solubilization of essential nutrient such as phosphate (Ahemad et al., 2014), increased acquisition of iron by microbial siderophore (Tang et al., 2015), resistance to adverse conditions like draught and freezing by microbial trehalose (RodrÃ-guez-Salazar et al., 2009) and antifreeze proteins (Duman et al., 1993).

1.2.3. PGPB in the aquatic environment

In aquatic environment, the growth promotion mechanisms are not necessarily the same as soil-based plants. While in soil most of the microorganisms are recruited in the rhizosphere of the plant through a chemical signal gradient (Chagas et al.,

2018), such kind of a chemical gradient is not reasonable in water bodies by aquatic macrophytes like duckweed. It is confirmed that IAA, siderophore and HCN production by bacteria has no statistical correlation with duckweed PGP (plant growth promotion) activity and phosphate solubilization has a very weak positive correlation (Ishizawa et al., 2017a). Moreover, our laboratory experiments have suggested that application of exogenous plant hormones such as 0.1-1 µM plant hormones (IAA, Gibberellin, Salicylic acid, Jasmonic acid, Abscisic acid) have no or negative effect on growth of *L. minor* (Utami et al., 2018).

1.2.4. Duckweed growth promoting bacteria

Studies on microbial communities and their interaction with aquatic plants such as duckweed has commenced recently (Ishizawa et al., 2017b; Ishizawa et al., 2019). Using duckweed-microbe co-cultivation method, taxonomically and functionally novel microbes have been isolated which otherwise wouldn't be found by culture methods (Yamaga et al., 2010). One such bacteria is phenol degrading *Acinetobacter calcoaceticus* P23 which was isolated from the rhizosphere of duckweed (Suzuki et al., 2014). Interaction of P23 strain with duckweed elucidates one of the mechanisms of how epiphytic bacteria interact with host plant through production of novel polysaccharides. Phenolic compound degradation by duckweed-microbe co-cultivation method has also been reported (Kristanti et al., 2012; Yan Li et al., 2014). Ease of axenic cultivation and availability of genome information makes it an attractive platform for studying plant-microbe interactions and the underlying mechanisms of microbe-induced growth promotion and inhibition effects on vascular plants (Ishizawa et al., 2019). Recently, community composition and methane oxidation activity of methanotrophs associated with duckweeds showed that duckweed can be inhabited by methane oxidizing communities and can stimulate bacterial growth that can be utilized in the future for efficient waste water treatment (Iguchi et al., 2019). It is evident that co-culture between duckweed and bacterial communities or single bacterial culture can have a promotional effect on the growth of associated bacteria or the host duckweed alone or the growth of both these

organisms in synergy. Utilization of such studies for maximizing the production of duckweed biomass in wastewater or other polluted water bodies with the help of duckweed growth promoting bacterial communities has a tremendous potential for being a keystone in large scale sustainable biomass production for the future. Moreover, isolation of PGPB (Plant growth promoting bacteria) from rhizosphere can further be used as a PGPB for terrestrial plants. One such example is a duckweed PGPB *A. calcoaceticus* P23 strain that also promoted the growth of a dicot *Lactuca sativa* (Suzuki et al., 2014). Interestingly, these PGPBs can be used on the duckweed itself to increase the growth of biomass yield significantly without the addition of external energy which can prove useful for making sustainable industries in the future.

1.2.5.Bacterial extracellular polymeric substances (EPS)

EPS are hygroscopic natural polymers produced and secreted by a myriad of prokaryotes and algae (Staudt et al., 2004). Usually made up of polysaccharides, EPS can also consist of other macromolecules such as DNA, lipids or humic substances. Monomeric sugar composition, structural characteristics of EPS defines the physicochemical characteristics of the microbial biofilm (Evans et al., 2005). Production of EPS may provide bacteria with a range of benefits including increased nitrogen fixation (Wang et al., 2017) and metal resistance (Van der Lelie et al., 2020), maintain nutrient balance (Zhang et al., 1994). In the soil, bacterial EPS and biofilm matrix mediates the plant root –bacteria interaction (Flemming et al., 2010). In some cases EPS formation is essential for root colonization of soil bacteria including endophytes (Flemming et al., 2016; Meneses et al., 2011). Secondary metabolites from halotolerant bacteria may act as osmoprotectant for plants which originate from their bacterial mutualistic partners, alleviating osmotic stress of the plant (Dodd et al., 2012). Such compounds can control stomatal movement and transpiration (Paul et al., 2014; Saghafi et al., 2019). Increase of plant biomass thorough the increase of plant growth hormones and higher root nodulation enhanced by the rhizobial exopolysaccharides as supplement has also been reported (Tewari et al., 2020). *Mesorhizobium loti*, which is a natural symbiont of Lotus produces monomeric octasaccharides called

as R7A EPS that act as signaling molecule for growth promotion of the host (Wong et al., 2020). EPS can further provide heavy metal resistance to plants by ameliorating the toxic effect of Zn in Maize (Jain et al., 2020), Cu in *Phragmites* (Kunito et al., 2001). Salt tolerant rhizobacteria can provide salt tolerance by absorbing Na+ from the plant growth medium, ultimately promoting the associated plant's growth (Tewari et al., 2014; Upadhyay et al., 2011; Yang et al., 2016).

1.2.6. Effect of EPS on duckweed

Duckweed has been used as a model for eco-toxicology studies (Forni et al., 2015). As duckweed is not rooted in soil, constant water flow and substrate diffusion has a role in diminishing growth promotion effect as well as phytoremediation efficiency. Production of EPS by a PGPB maybe one of the most important factor for bacterial colonization on duckweed, which may lead to creating more efficient waste water phytoremediation strategies (Ishizawa et al., 2017b, 2019; Khairina et al., 2021). *Acidobacteria* isolated from wild duckweed *S. polyrhiza* was observed to have formed network structures likely composed of EPS on the roots of the host (Yoneda et al., 2021). Based on the remarkable adsorption quality of the EPS, a tri partite bio-hybrid material has been made where oil degrading bacteria were immobilized and kept biologically viable with the nutrient provided by duckweed (Lobakova et al., 2016). Elucidation on how bacterial EPS from PGPB effect the duckweed, new biotechnological path may be opened.

1.3. Introduction to *Azotobacter vinelandii*

A. vinelandii is a gram-negative motile γ-proteobacterium. This obligate aerobic (Ackrell & Jones, 1971) diazotroph (Premakumar et al., 1984) forms desiccation resistant cysts (Wyss et al., 1961) and polyhydroxyalcanoates (PHA) as storage polymers (Page et al., 1992). Wild type cell morphology is pleomorphic, varying depending on the physiological state and growth conditions.

1.3.1. Applications of *A. vinelandii*

1.3.1.1. Polyhydroxyalkanoate (PHA) production

Azotobacter vinelandii produces alkyl polyesters, PHA (polyhydroxyakanoate) and PHB (polyhydroxybutyrate) in certain conditions. PHB produced by *A. vinelandii* (Forsyth et al., 1958) during the cyst formation phase are a form of renewable bioplastics (Akaraonye et al., 2010), are biodegradable (Mergaert et al., 1992) and they can be used as raw material for biocompatible medical devices (Ivanov et al., 2015) or cell culture scaffolds (Romo-Uribe et al., 2017). 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.

1.3.1.2. Production of 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. Alginates are products of interest industrially as they can be used as stabilizing, gel-forming or thickening agents in various processes in the food, pharmaceutical and cosmetics industries (Rehm et al., 1997; Yao et al., 2010)

1.3.1.3. *A. vinelandii* as a PGPB

Azotobacter vinelandii has been frequently reported for increasing growth and yield of terrestrial plants through non-symbiotic dinitrogen fixation (Wilson et al., 1990), phytohormone, especially cytokinin production (Abbass et al., 1993; Danapriatna et al., 2013; Taller et al., 1989) and exopolysaccharides production (Giti et al., 2004; Vermani et al., 1997). Phosphate solubilizing activity of *Azotobacter* sp. on agriculture has also been reported (Kumar et al., 1999). Finally, application of *Azotobacter* sp. at 3 L ha^{-1} provided maize with a production yield comparable to inorganic fertilizers (Katriani et al., 2011). It is chosen for this experiment because it is hypothesized that a nitrogen fixing PGPB that not only

fixes nitrogen in nutrient poor wastewater but also produces growth promoting substances for the duckweed for maximizing biomass can open the door to many sustainable applications.

1.3.1.4. *A. vinelandii* and photosynthetic host symbiotic co-cultures

The nitrogen fixing capacity of *A. vinelandii* has been utilized to study bacterial co-culture experiments with photosynthetic organisms. *Daucus carota* and nitrogen-fixing *Azotobacter* cells in vitro can form an artificial association in nitrogen free medium (Varga et al., 1994). One such experiment shows that green algae *Neochloris oleoabundans* and *Scenedesmus* sp. BA032 can utilize the *A. vinelandii* siderophore azotobactin as a source of nitrogen while providing carbon source for the bacterial cells by photosynthesis indicating a communalistic relationship (Villa et al., 2014). Genetically modified *A. vinelandii* cells have also been reported to benefit the growth of both microalgae and cucumber in separate co-culture experiments (Ambrosio et al., 2017). *A. vinelandii* can also form tripartite symbiosis with a green alga *Chlamydomonas*, a fungus *Alternaria* (Lőrincz et al., 2010).

1.3.2. Nitrogen fixation ability of *A. vinelandii*

The nitrogenase enzymes of *A. vinelandii* are well characterized (Chisnell et al., 1988; Premakumar et al., 1984). The dinitrogen reduction process begins when the nitrogenase or DNR (dinitrogen reductase) consumes two ATP molecules required for the reaction from N_2 (Hageman et al., 1980). Nitrogenase can also reduce acetylene (C_2H_2), azide (N_3^-) and cyanide (CN^-) (Seefeldt et al., 1995). Nitrogenase activity is completely stopped by NH_4^+ at 25µM or above (Kleiner et al., 1974). Nitrate salts, organic compounds such as aspartate, adenine, yeast extract or casamino acids can also repress nitrogenase partially (Gadkari et al., 1974; Horner et al., 1944). Aside from nitrogen fixation, *A. vinelandii can* also use inorganic sources (ammonium, nitrate, nitrite) and several organic sources: aspartate, asparagine, glutamate, adenine and urea for growth (Horner et al., 1944).

1.3.3. EPS of *A. vinelandii*

Azotobacter have been reported to produce alginate as its primary constituent of the EPS. This biotechnologically important EPS has many applications as bio-sorbent. Cr and Cd in contaminated soils have been remediated with *Azotobacter* EPS (Joshi & Juwarkar, 2009) while The EPS of *Azotobacter chroococcum* XU1 is capable of absorbing, significant amount of Pb and Hg from aqueous solution (Rasulov et al., 2013). *Azotobacter* EPS can act as a Carbon source or a carbon sink depending on medium nitrogen availability (Otero & Vincenzini, 2003) which may eventually positively promote the growth of plants. *Azotobacter* EPS alginate is polymer made up of a (1–4)-linked β-D-mannuronic acid and its C5-epimer α-L-guluronic acid which can also be called alginic acid (Figure 3). These sugar acids are distributed in blocks of continuous β-D-mannuronic acid residues (M-blocks) and α-L-guluronic acid residues (G-blocks), or as alternating residues (MG-blocks) (Rehm et al., 2009). There are acetylation at the positions O-2 and /or O3 and sequential epimerization. *AlgA, AlgC, AlgD, AlgE, AlgG, AlgL, AlghJ, AlgF* are the genes responsible for biosynthesis and secretion of alginate.

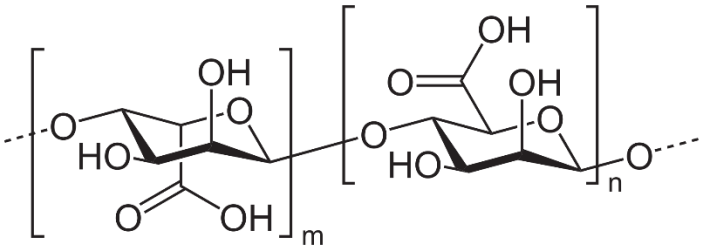


Figure 3: Alginic acid structure. Alginic acid is composed of α-L-guluronic acid and β-D-mannuronic acid residues.

Alginate biosynthesis and regulation genes (Pacheco-Leyva et al., 2016) are summarized in a Table 1 below:

Table 1. Alginate biosynthesis genes adopted from (Pacheco-Leyva et al., 2016)

Gene	Protein	Function
*alg*A	AlgA :Phosphomannose isomerase	Synthesis of fructose-6-phosphate to mannose-6-

	(PMI)/guanosine-diphosphomannose pyrophosphorylase (GMP)	Phosphate, and conversion of mannose-1-phosphate to GDP-mannose.
*alg*C	AlgC: Phosphormannomutase	Directly converts the mannose-6-phosphate into mannose-1-phosphate
*alg*D (Limiting step)	AlgD: GDP-mannose dehydrogenase	Oxidation of GDP-mannose to GDP-mannuronic acid
*alg*8	Alg8: Glycosyltransferase	A component of the core of the polymerase complex that transports alginate polymer precursors across the cytoplasmic membrane.
*alg*44	Alg44: Glycosyltransferase	Same as Alg8
*alg*k	AlgK	Stabilization of the polymerase complex Alginate doesn't form in absence of AlgK
*alg*U	AlgU: Alternative sigma factor σ^E	Regulate the expression of functions related to the extracytoplasmic compartment
*alg*T	AlgT: Alternative sigma factor σ^E	
*alg*I	AlgI, AlgV, AlgF, AlgX Acetylase enzymatic complex	O-acetylase modification
*alg*V		
*alg*F		
*alg*X		
*alg*G	AlgG: Epimerase	Epimerization of non-o-acetylated M-residue: Poly(β-d-mannuronate) to α-l-guluronate High o = low epimerization
*alg*L	AlgL: Bifunctional mannuronan C-5 epimerase AKA alginate lyase	Depolymerization at the 4-O-glycosidic bond via β-elimination

*alg*E7	AlgE7: Alginate lyase	Depolymerization at the 4-o-glycosidic bond via β-elimination
*aly*A(1–3)	Lyases	Uncharacterized
*aly*B	Exolyase	Uncharacterized
*alg*E	Porin	Outer membrane secretin
*alg*G, *alg*K, and *alg*X	Scaffold complex	Scaffold complex for transporting recently modified polysaccharide to the extracellular milieu.

2. Diazotrophic bacterium *A. vinelandii* as a mutualistic growth promoter of an aquatic plant *Lemna minor*

2.1. Introduction

Duckweed, family Lemnaceae, is a small floating aquatic plant, capable of growing ubiquitously and rapidly absorbing nutrient minerals from water under various climate conditions. Thus, duckweed is considered as a competent tool for energy saving wastewater treatment system (Bonomo et al. 1997; Yamaga et al. 2010) and subsequent biomass production (Xu et al., 2011). Duckweed biomass has been used not only as an animal feed but also as human food (Leng et al., 2004) due to its high protein content and other nutritional values (Appenroth et al. 2017). When grown under stressed condition, the duckweed decreases protein production level and in turn accumulates high amount of starch, which then can be also applied as feedstock for biofuel production (Su et al., 2014; Toyama et al., 2018). Improvement of the wastewater treatment and duckweed biomass production technologies by utilizing duckweed associated bacteria or aquatic microbial community is being recently spotlighted as a promising eco-friendly biotechnology (Ishizawa et al. 2017b, 2020; Khairina et al. 2021; Yamaga et al. 2010). It has been reported that a duckweed surface associated *Acinetobacter calcoaceticus* P23 significantly promotes the growth of *Lemna minor* not only in a gnotobiotic medium but also environmental water or treated sewage effluent (Toyama et al. 2017; Yamaga et al., 2010). While *A. calcoaceticus* P23 predominantly colonizes on the fronds (a leaf like structure of duckweed), *Pseudomonas fulva* Ps6 mainly colonizes on the roots and similarly promotes the growth of *L. minor*. It was demonstrated that the duckweed/bacteria co-culture system can be a model platform for studying multiple interactions among host plants and the associated bacteria such as P23 and Ps6 (Yamakawa et al., 2018).

Several studies have reported that aquatic macrophytes can be benefitted, for example, by bacterial nitrogen fixation (Biesboer etr al., 1984; Hay et al., 2004; Rivas et al., 2003) , allelopathic activities against algae and cyanobacteria (Hempel et al. 2008; Rao et al. 2006), indole acetic acid (IAA) production (Halda-Alija, 2011). The mechanisms of plant growth promotion of duckweeds by

bacterial strains are now being studied further in detail (Gilbert et al. 2018; Idris et al. 2007; Ishizawa et al. 2017a; Toyama et al. 2022; Utami et al. 2018). Nitrogen is one of the major essential minerals for plant growth. Free-living nitrogen fixing bacteria such as cyanobacteria (Duong et al.,1985), *Klebsiella* and unclassified aerobic diazotrophs have been reported to associate with duckweed mats in ponds where they could provide about 15-20% of the duckweed nitrogen requirement through biological nitrogen fixation (Zuberer et al., 1982). *Azotobacter*, a dominant group of free-living soil diazotrophs that has been frequently reported for promoting the growth and yield of terrestrial plants through not only non-symbiotic dinitrogen fixation (Sprent et al., 1990) but also phytohormones, cytokinin production (Abbass et al., 1993; Taller et al., 1989) and exopolysaccharides production (Giti et al., 2004; Vermani et al., 1997). The beneficial effect of phosphate solubilizing activity of *Azotobacter* bacteria on agriculture has been also reported (Kumar & Narula 1999). Inoculation of *Azotobacter* bacteria showed 23% higher grain productivity in maize when compared to inorganic fertilizers (Mahato et al., 2018). Considering that duckweed is a group of terrestrial plants, we were thus interested if *Azotobacter vinelandii* could co-exist and affect the growth of gnotobiotic *L. minor* in aquatic condition. A both side beneficial symbiotic, so called mutualistic, association between host plant *L. minor* and guest bacterium *A. vinelandii* is expected to bolster the growth of both organisms and result in a higher duckweed biomass yield in extremely nutrient poor such as nitrogen- and carbon-free water conditions, leading to the expansion of its application sites for duckweed biomass production.

2.2. Aim and objectives

i. Establishment of an artificial mutualistic relationship between *L. minor* and *Azotobacter vinelandii* A81

ii. Quantification and characterization of the factors affecting mutualistic plant growth promotion effect and the long-term survivability of both partners

2.3. Materials and Methods

2.3.1. Bacterial strains, plants, and culture conditions

2.3.1.1. Bacterial strains

A typical free-living diazotrophic bacterium *Azotobacter vinelandii* ATCC 12837= NBRC 13581 (hereafter, A81) was mainly used in this study. A nitrogenase gene deletion mutant (delta-nifHDK) *A. vinelandii* CA12 (Bishop et al., 1986) was kindly provided by Professor Dr. Markus W. Ribbe, Department of Chemistry, University of California Irvine (Lancaster et al., 2011). *A. vinelandii* CA12 is developed from its parent strain *A. vinelandii* CA (ATCC 13750), a type strain. *A. vinelandii* CA was collected from ATCC for this research. Three plant growth-promoting bacteria, PGPB, for duckweed were used for reference bacterial strains. *Acinetobacter calcoaceticus* P23 (Yamaga et al. 2010) and *Pseudomonas fulva* Ps6 (Yamakawa et al. 2018) were previously isolated from a natural duckweed, *Lemna aoukikusa* and *Lemna minor* RDSC 5512, respectively, native to a pond in Hokkaido University botanic garden, Sapporo, Japan. *Ensifer* sp. SP4 was isolated from a duckweed *Spirodela polyrhiza* cultured in municipal wastewater effluent, Kofu, Japan (Toyama et al., 2022).

2.3.1.2. Culture conditions

All the bacteria were stored at -80°C in cryotube. Liquid and solid agar of Luria (L) broth (Bertani, 1951) and Burk's nitrogen free (BS) medium (Strandberg et al., 1968) were used as culture media for *A. calcoaceticus* P23, *P. fulva* Ps6, *Ensifer* sp. SP4, and *A. vinelandii* A81, respectively. *A. vinelandii* CA12 was grown in BS medium supplemented with 2.25 g/L ammonium acetate (CH_3COONH_4) for nitrogen source. Bacterial fresh culture was prepared each time by shaken incubating at 30 °C for 2 or 3 d depending on the growth.

2.3.1.3. Plant culture conditions

L. minor RDSC 5512 was previously sterilized by sodium hypochlorite treatment and aseptically maintained in the laboratory (Suzuki et al., 2014). Culture condition of *L. minor* was 28°C, 60% relative humidity, 5,000 lx (75 µmol m^{-2}

s^{-1}) illumination, 16 h-photoperiod in modified Hoagland, mH, medium (Suzuki et al., 2014). Other duckweed species *Lemna gibba* G3 RSDC 362, *Wolffiella hyalina* (provided by Graduate School of Science, Kyoto University, Oyama laboratory), *S. polyrhiza* (provided by Graduate Faculty of Interdisciplinary Research, University of Yamanashi, Toyama Laboratory) were also kept in an aseptic stock and cultured in the same condition. Sterility of duckweed was confirmed by no bacterial colony formation on L agar plate after incubation at 30°C for 3 d.

2.3.2. Duckweed growth promotion experiments

2.3.2.1. Plant growth promotion assay

Two different co-culture condition was used in this experiment. For "suspension experiment", fresh bacterial culture was prepared and centrifuged at 7,700 × g for 10 min at 4°C to retrieve the bacterial cells as pellet. The pellet was washed with mH medium, re-suspended, and diluted to make a uniform bacterial cells suspension of 0.3 OD_{600} (about one million cells) in 50 ml mH medium, where two plant bodies (two fronds with two roots) from aseptic duckweed stock was placed and co-cultured under plant growth condition. For "attachment experiment", two plants of *L. minor* were cultured in bacterial cells suspension as above described for 48 h followed by rinsing the plants by submerging them twice in sterilized distilled water followed by introducing them into bacteria free 50 ml mH medium for co-culture. Similar experiments were also conducted in 50 ml nitrogen-free mH-N medium, where KNO_3 was replaced by K_2SO_4, and BS medium if necessary. All the experiments were done in three replications (n=3) for statistical analysis. The number of fronds, leaf like structure, were counted every 2 d until 10 d and final colony forming units, CFU of bacterial cells and dry weight of plants were measured.

2.3.2.2. Measurement of protein content

L. minor was harvested, and fresh weight was measured before vacuum freeze drying (FDU 1110, EYELA, Tokyo, Japan). Protein fraction was prepared using Apro science protein extraction kit (Naruto, Japan) and quantified with D_C protein

assay kit (Bio-Rad, Hercules, CA). Protein contents, % protein/dry weight was estimated using standard curve made by different concentration of BSA (0.125 mg/ml to 2 mg/ml).

2.3.2.3. Measurement of starch content

L. minor was harvested, and fresh weight was measured before freeze drying in vacuum. The freeze-dried biomass was used for measuring the starch contents, % starch/dry weight with Megazyme total starch assay kit (NEOGEN, Lansing, MI).

2.3.2.4. Chlorophyll content enumeration

Chlorophylls were extracted by 1 mL of cold ethanol saturated with $Ca(CO_3)_2$ using 0.5 g glass beads (φ 0.1 mm) and a multi bead shocker [MB755U(S), Yasui Kikai, Japan] at 2500 rpm for 60 s (4°C). Chlorophylls present in the centrifuged clear extracts were quantified by measuring photometric absorption at 649 nm and 665 nm (Ritchie, 2006). The chlorophyll content was determined by mg chlorophylls/100 g wet weight of frond or leaf specimens.

2.3.3. Identification of bacterial plant growth-promoting factors

2.3.3.1. Biofilm formation assay

A 400 µL of L medium was inoculated with 1% pre-culture in 1.5 ml Eppendorf tube and kept standing at 30°C up to 5 d. After measuring the OD_{600}, planktonic cells in liquid culture was carefully removed by micropipette. After rinsing the wells with 500 µL MilliQ water 600 µL of 0.1% crystal violet, CV, solution was added and kept at room temperature in dark for 20 min. CV solution was removed and rinsed with MilliQ water three times. CV dye bound with biofilm was extracted by 600 µL of 33% acetic acid. Amount of biofilm was estimated by measuring the OD_{535} of CV.

2.3.3.2. IAA (Indole acetic acid) production assay

Bacterial fresh culture was prepared by shaking incubator for 24 h at 30°C in 5 ml of liquid L medium in glass test tubes in the presence and absence of 200 mg/L

tryptophan for IAA production assay (Gordon & Weber, 1951). Bacterial culture was centrifuged at 4,000 x g at 4°C for 15 min. Supernatant was diluted 50% with MilliQ water and 200 µL Salkowski's reagent (a mixture of 50 ml of 35% perchloric acid and 1 ml of 0.5 M $FeCl_3$) was added. After kept in dark for 25 min, OD_{530} was measured. IAA production (µg/mg biomass) was quantified using standard curve made by IAA (5 µg/ml-100 µg/ml).

2.3.3.3. Phosphate solubilization assay

Bacterial fresh culture was prepared by incubating bacteria in 5 ml of liquid L medium in shaking incubator at 100 RPM at 30°C for 24 h. Pikovskayas agar plate (Pikovskaya 1948) was inoculated with a drop of 10 µL of fresh bacterial culture and incubated at 30° C for 48 h. Diameter of the clear halo around the bacterial colony was measured and expressed as the phosphate solubilization activity of the strains.

2.3.3.4. Siderophore production assay:

The assay was conducted using chrome azurol S, CAS (Schwyn & Neilands 1987) both qualitatively and quantitatively. The blue color is attributed to the CAS/HDTMA that is tightly bound with ferric iron. A strong iron chelator such as a siderophore can remove iron from the dye complex. This changes the color from blue to orange. Fresh bacterial culture was prepared at 30° C for 48 h in 1.5 ml tube containing 1.0 ml L medium. The bacterial culture was centrifuged at 7,700 × g 4°C for 10 min and 0.5 ml of supernatant was mixed with 0.5 ml of CAS reagent. After 20 min, absorbance at 630 nm was measured using spectrophotometer. The percent siderophore units (psu) was calculated using a mathematical formula (Arora & Verma, 2017). For qualitative detection of siderophore, a modification of simple double-layered CAS agar (SD-CASA) diffusion assay was used (Hu & Xu, 2011). Bacterial colonies were grown in L agar media for 48 h were overlaid with 10% CAS in 1% molten agar solution and incubated at 30°C for additional 48 h or until orange halo formation was observed around the colonies in blue background in positive control.

2.3.3.5. Nitrogen fixing activity

Nitrogen fixing activity was measured by acetylene reduction assay (ARA) under plant colonized and bacterial suspension conditions. ARA is based on the activity of nitrogenase enzyme to catalyze the reduction of acetylene to ethylene (Bergersen, 1970). The quantitative relationship between the reduction of N_2 to NH_3 and simultaneous reduction of C_2H_2 to C_2H_4 by the nitrogenase enzyme is consistent and have been extensively used to study the nitrogen fixation activity of root nodule forming bacteria. Given that the substrate, energy supply, and reductant supply are not limiting, the ratio of the products for equal numbers of electrons transferred can be assumed to be 1.5:1 according to the reactions:

$3C_2H_2 + 6H^+ + 6e^- \rightarrow 3C_2H_4$,

$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$.

Alternatively, it can be expressed that the ratio of nitrogen to acetylene reduced is 3:1. When nitrogen fixing bacteria fix nitrogen, any C_2H_2 in the airtight reaction vial will be reduced to C_2H_4. The amount of ethylene (C_2H_4) produced in the headspace of the 5ml glass vial is measured by GC-2014-FID (Shimadzu, Kyoto, Japan) with a Shincarbon-ST 50/80 mesh (4.0 m x 3.0 mm ID stainless column, Shinwa Chemical Ind. Ltd., Tokyo, Japan). The standard curve of peak area vs amount ethylene was made by increasing injection volume of 803 ppm ethylene to GC.

2.3.3.5.1. Bacteria colonized on *L. minor*

ARA of bacteria on *L. minor* was conducted using a method described previously (Zuberer, 1982) with several modifications. Briefly, *L. minor* and *A. vinelandii* A81, CA12, or *Ensifer* sp. SP4 co-culture was prepared in 0.3 OD_{600} bacterial suspension in nitrogen free medium mH-N and incubated for 2 d under plant growth condition. After gentle washing with sterile MilliQ water to rinse out loosely attached bacterial cells, initial amount of colonized bacterial cells was measured by colony forming units, CFU. A 3 g fresh weight of *L. minor* colonized by bacteria was put and sealed airtight with butyl rubber stopper and aluminum crimp in a 5 ml glass vial without liquid medium. Control vial containing aseptic *L. minor* was also prepared. Headspace gas was replaced 10% with acetylene and

incubated 5 d for ARA under plant growth condition. Final CFU of colonized bacteria on *L. minor* was also measured. For measuring the CFU, aliquots of plant bodies were mashed with Nippi Biomasher II, Tokyo, Japan and serial dilutions of the sample were enumerated on L plate by spread plate technique. This process was also done for the bacteria-free control experiments to verify no bacterial contamination.

2.3.3.5.2. Bacteria in suspension co-cultured with *L. minor*

ARA of *A. vinelandii* A81 and CA12 in suspension co-cultured with or without *L. minor* was conducted using a method described by Bergersen 1970 with several modifications. Aseptic *L. minor* of 5 g was placed in a 5 ml glass vial and inoculated with 2 ml bacterial cell suspension of 1.0 OD_{600}, 0.8 x 10^9 CFU/ml in nitrogen free BS medium. Control vials were also prepared that contained only bacterial suspension or *L. minor*. The glass vials were closed with aeration cap (Silico-sen, Shin-etsu chemical Co. Ltd., Tokyo, Japan) and kept standing for 6 h in plant culture condition. After measuring CFU, the glass vials were closed airtight followed by conducting ARA at 30°C for 1 h.

2.3.4. CFU measurement

For measuring the CFU, at least two frond/roots were taken from each replication and a total of 6 fronds/3 roots were mashed with Nippi Biomasher II, Tokyo, Japan and s 10^3 fold serial dilutions of the sample were enumerated on L agar and BS agar by spread plate technique. This process was also done for the bacteria-free control experiments to verify no bacterial contamination.

2.3.5. Scanning electron microscopy (SEM)

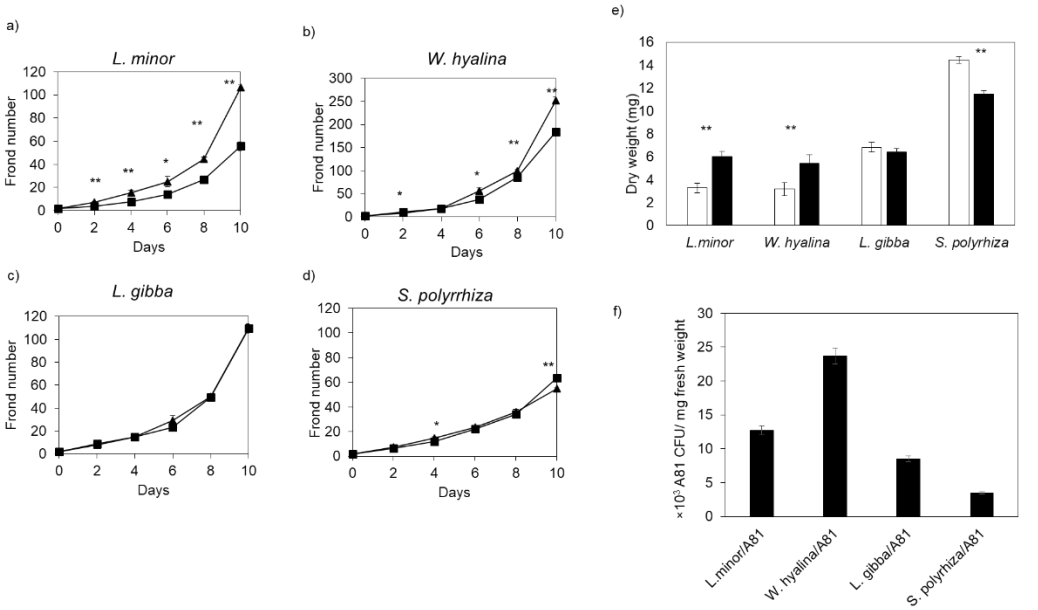
Sample of bacteria colonized on either polypropylene or *L. minor* was fixed with 5% OsO_4 followed by 2% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.0). After fixation, the samples were dehydrated by stepwise increasing concentration of ethanol followed by treatment of critical point carbon dioxide.

Specimen was sputter coated with gold and observed by a Model S-2400 (Hitachi, Tokyo, Japan) scanning electron microscope.

2.4. Results

2.4.1. Plant growth-promoting activities of *A. vinelandii* A81 in mH medium

The PGP activity was examined by suspension experiments against four different Lemnaceae plants. Among the plants tested in gnotobiotic A81 co-culture



condition for 10 d of incubation, *Lemna minor* and *Wolffiella hyalina* showed apparent increased frond numbers up to 1.5 and 1.3-folds and dry weight up to 1.7 and 1.6-folds, respectively, compared to no bacterial control (Figure 4. a-e). It was also found that A81 successfully colonized on all the duckweed species, *L. minor*, *W. hyalina*, *L. gibba*, *S. polyrhiza* (Figure 4. f). Relatively higher and lower CFU vs fresh weight values in *W. hyalina* and *S. polyrhiza* were probably due to their different surface area per fresh weight. We chose *L. minor* for further experiments as a model plant.

Figure 4. Effect of *A. vinelandii* A81 inoculation on the growth of four different duckweeds in mH medium. Increase in the frond numbers is shown for (a) *L. minor*, (b) *L. gibba*, (c) *W. hyalina*, and (d) *S. polyrhiza*. All the duckweed culture experiments were started from two fronds, plants. Symbols are triangle, co-culture with *A. vinelandii* A81;

square, no bacteria control. (e) Growth of each duckweed was compared by dry weight after 10 d. Open bars, dry weight of duckweed without bacteria; closed dry weight of duckweed co-cultured with A81. (f) CFU of *A. vinelandii* A81 recovered from the duckweed co-cultured for 10 d. Values are mean ± SD (n = 3). Asterisks indicate the significant differences between values with and without A81 (Student's t-test, * $P < 0.05$, ** $P<0.005$).

2.4.2. Growth recovery of *L. minor* by *A. vinelandii* A81 in nitrogen-free mH-N medium

The ability of A81 to recover the plant growth in no nitrogen containing water condition (mH-N) was examined. In order to minimize the effect of bacterial cell suspension as simple nutrient source, the PGP effect of only the duckweed colonized bacteria was examined by the attachment experiment described in 2.2.2.1. It revealed that the *L. minor* colonized by A81 successfully increase the frond number and dry weight by 2.0 and 2.2-folds respectively after 10 d, compared to the nitrogenase genes, *nifHDK* deletion mutant CA12 and no-bacteria control (Figure 5). CFU of A81 and CA12 in the liquid medium after 10 days was 18.73×10^3 and 0.03×10^3 per plant, respectively. Change in CFU of A81 was further observed for 30 d by transferring A81 colonized *L. minor* plants to new flask every 10 d. CFU was similarly increased on the fronds and roots in mH medium (Figure 6a). On the other hand, increase in CFU was much more on the fronds than the roots in mH-N medium (Figure 6b). Significant increase in CFU of A81 on the plant for 30 d and its PGP effect demonstrates a stable mutualism constructed between the guest bacteria and the host plant. In the same experiment, CA strain showed similar PGP effect on *L. minor* as A81 (figure S1). The PGP effect of the CA12 in mH medium (with nitrogen) is also shown in the appendix (figure S1).

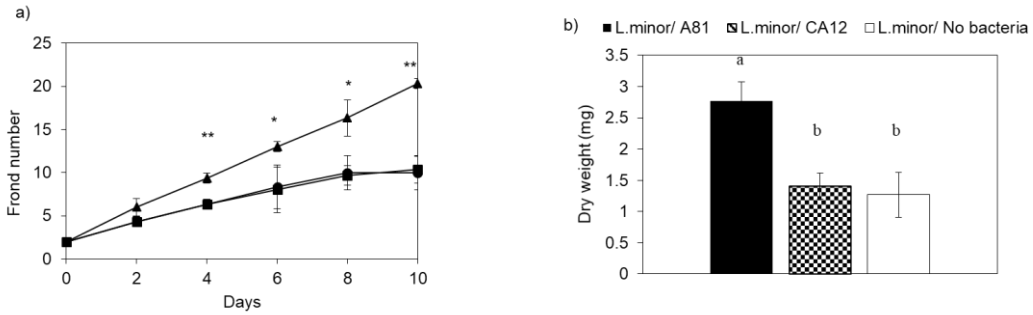


Figure 5. Effect of *A. vinelandii* A81 and CA12 on the growth of *L. minor* in nitrogen-free medium, mH-N. (a) Increase in the frond numbers. Symbols are triangle, *A. vinelandii* A81 co-culture; circle, *A. vinelandii* CA12 co-culture; square, no bacteria. (b) Dry weight of *L. minor* after 10 d. Closed bar, with A81 co-culture; checkered bar, with CA12 co-culture; open bar, no bacterial control. All values are mean ± SD (n = 3). Asterisks indicate the significant differences between values with and without bacteria (Student's t-test, * $P < 0.05$, ** $P<0.005$). Different alphabets indicate significant differences.

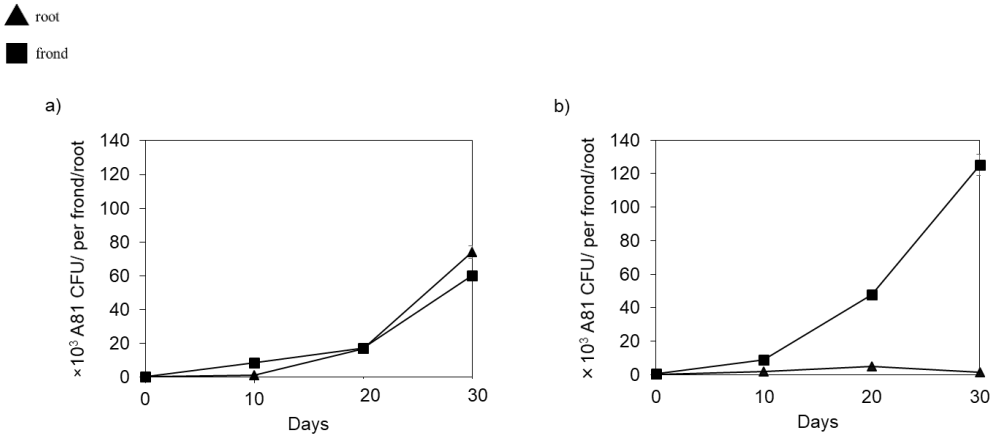


Figure 6. Change in the CFU of A81 colonized on *L. minor* for 30 d, when cultured in (a) mH and (b) mH-N (nitrogen-free) media. CFU was separately counted for frond and root parts before each transfer on day 0, 10, 20. Symbols are triangle, CFU on one root; square, CFU on one frond. All values are mean ± SD (n = 3).

2.4.3.Nitrogen fixing activity of *A. vinelandii* A81

2.4.3.1. Bacteria colonized on *L. minor*

L. minor colonized by A81 produced 293.9 µmole ethylene/g fresh ethylene that is much more than bacteria-free *L. minor*, 123.5 µmole ethylene/g fresh (Figure 7). *L. minor* with nitrogenase negative strains, CA12 and SP4, only produced similar amounts of ethylene to the bacterial-free *L. minor*. Initial CFU of A81 and CA12 was 2.3×10^4 and 8.0×10^4 CFU/g fresh weight respectively while SP4 showed higher CFU of 7.0×10^5 CFU/g fresh weight immediately after 2d co-culture in mH-N. CFU of A81 was later increased from 2.3×10^4 to 5.0×10^5 after 5d on the *L. minor*, while producing 170.35 µmole ethylene/g fresh plant deduced from the bacteria-free control plants. On the other hand, the CFU of CA12 and SP4 were decreased or not much changed with negligible level of ethylene production 9.0 and 7.8 µmole ethylene/g fresh plant, respectively. These observations suggest nitrogen fixation was occurred by A81 colonized on *L. minor*. We observed ethylene production even in the control *L. minor* with no bacterial inoculation, which can be reasoned by *de novo* production of ethylene by the plant (Yang et al., 1984).

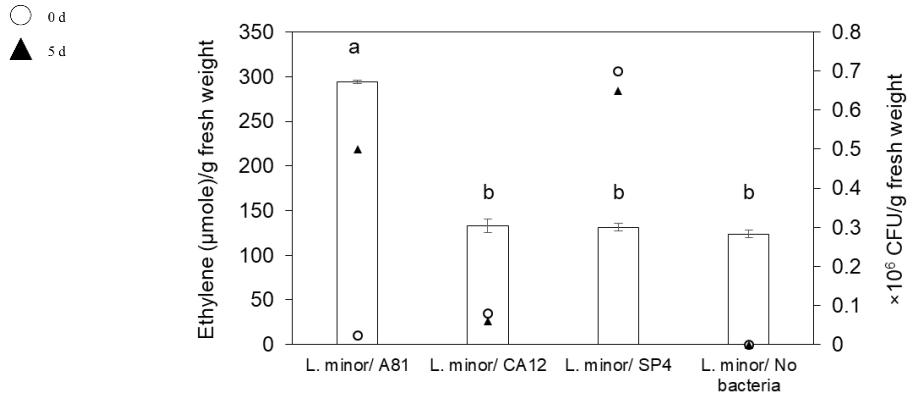


Figure 7. Nitrogen fixing activity of bacteria colonized on *L. minor*. Bars indicate amount of ethylene (µmole) detected in the experimental vial/g fresh weight. ARA was conducted for 5 d. Open circle, CFU of bacteria colonized on *L. minor* immediately after 2 d incubation in 0.3 OD_{600} bacterial suspension. Closed triangle, CFU of bacteria on *L.*

minor after 5 d. All values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test).

2.4.3.2. Bacteria co-cultured with *L. minor*

Nitrogen fixation by A81 was also indicated in a co-culture suspension experiment with *L. minor*. A81 cells produced higher amount of ethylene in the vials containing *L. minor*, 349.5 µmole/vial compared to vials without *L. minor*, 67.3 µmole /vial (Figure 8). CFU/vial of A81 was also higher when co-cultured with *L. minor*, 3.2 x $10^{9,}$ than without *L. minor*, 1.1 x 10^{9} after 6h suggesting positive effect by the plant. Only a small amount of ethylene was detected in the vial of CA12 co-cultured with *L. minor*, 2.2 µmole/h and without *L. minor*, 1.1 µmole/h. *de novo* production of ethylene by axenic *L. minor* was also in negligible level, 2.3 µmole/h.

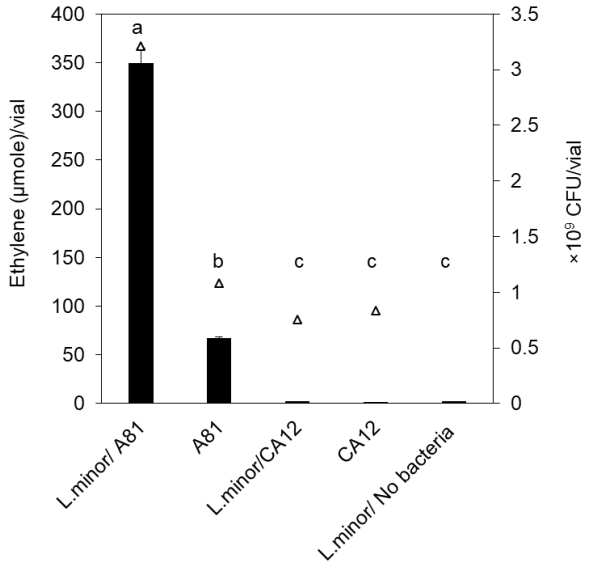


Figure 8. Nitrogen fixing activity of bacterial suspension co-cultured with *L. minor*. Open bars, Ethylene (µmole) detected/vial. Bacterial cells were inoculated at ca. 0.8 ×10^{9} CFU/ml BS medium except no bacteria control. After the pre-incubation of 6 h, the CFU was measured and ARA assay was conducted for 1 h. Open triangle, CFU values in the vial after 6 h pre-incubation. All values are mean ± SD (n = 3). Different alphabets between treatments indicate significant differences (one-way

ANOVA; $p < 0.05$, Tukey HSD as a post-hoc test). *L. minor* of 5 g was contained in each vial as indicated.

2.4.4. General plant growth-promoting factors produced by *A. vinelandii* A81

A. vinelandii A81 showed significant phosphate solubilization (0.4 cm halo outside of colony on Pikovskaya agar plate), siderophore production (24.0 ± 4.2 percentage siderophore unit: psu), and moderate IAA production activity (3.9±1.2 µg IAA/mg biomass). Summarized in Table 2.

Table 2. General plant growth promotion factors of the strains used in this study

Bacterium	Phosphate solubilization activity (cm)	Siderophore production Psu	IAA production (µg/mg biomass)	Biofilm formation OD_{595}
A. vinelandii A81 (ATCC 12837)	0.425	23.95±4.16	3.90 ±1.2	2.03
Acinetobacter calcoaceticus P23	0.34	47.08± 3.2	0.87 ±0.7	1.38
Pseudomonas fulva PS6	0.297	46.327± 3.2	17.59±1.86	0.23
A. vinelandii CA (ATCC 13705)	Not detected	21.23±9.35	9.03±0.93	0.065
A. vinelandii CA 12	Not detected	67.04± 2.2	8.33±0.97	0.372

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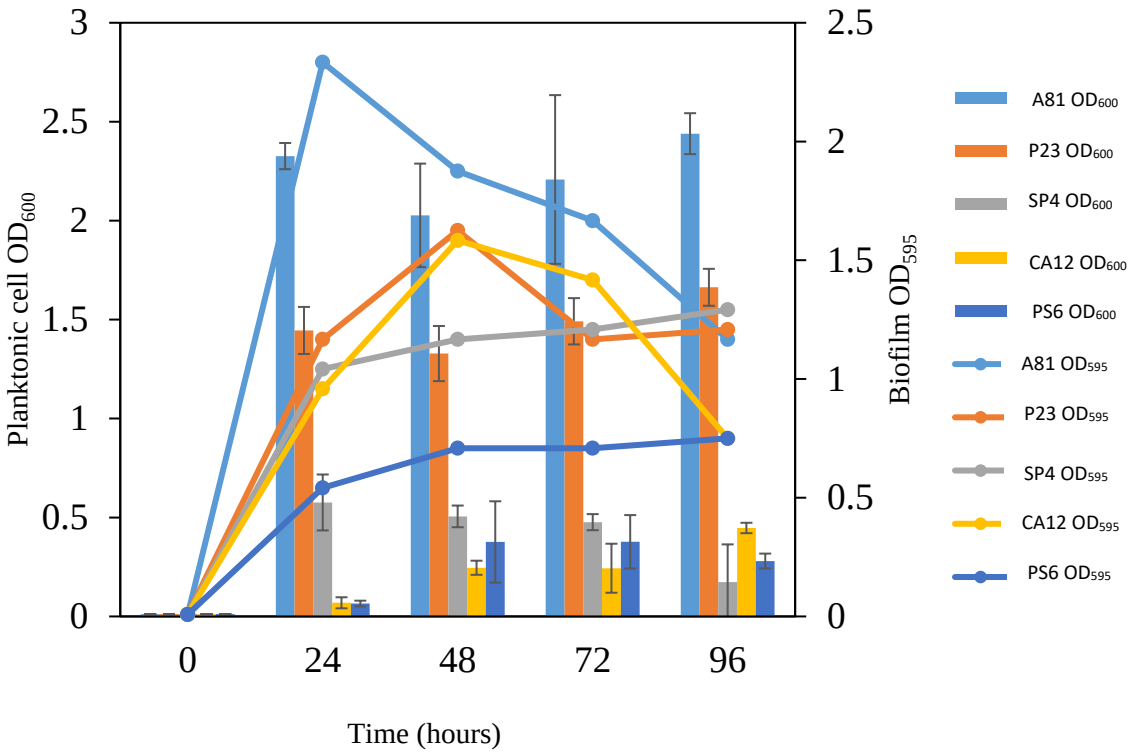


Figure 9: Biofilm production by various strains in this experiment. Lines of corresponding colors represent planktonic cell OD_{600}. Vertical bars of corresponding color represent biofilm OD_{595} stained by crystal violet. All values are mean ± SD (n = 3).

2.4.5. Effect of *A. vinelandii* A81 on the protein and starch contents in *L. minor*

It was found that the protein content in *L. minor* was increased upon co-cultured for 10 d in A81 suspension mH medium, 40.7± 2.2 %, compared to no bacterial control, 23.2± 3.4 %. In contrast to increase in protein content, starch content was dramatically decreased to 13.6± 4.2 %, compared to no bacterial control 48.9± 6.7% (Figure 10).

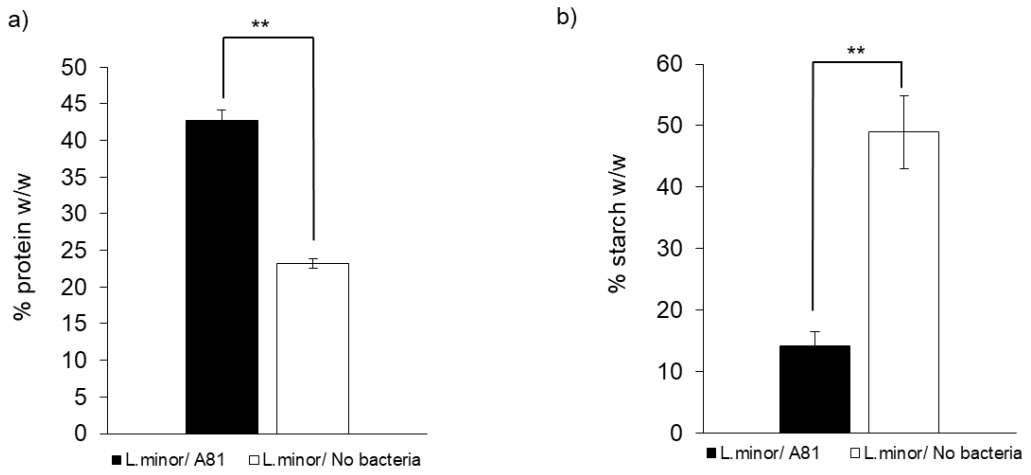


Figure 10. Effect of *A. vinelandii* A81 on the starch and protein contents of *L. minor* in mH medium. (a) % starch and (b) % protein of *L. minor* were measured after 10 d. Closed bars, *L. minor* co-cultured with A81; open bars, no bacteria (control). All values are mean ± SD (n = 3). Asterisks indicate the significant differences between values with and without bacteria (Student's t-test, * P < 0.05, ** P<0.005).

2.5. Discussion

Duckweed plant body provides an excellent nutritious residence and an ecological niche for bacterial cells in aquatic environments, especially when availability of organic carbon compounds is limited. A group of bacteria can actively proliferate and elicit growth promotion effect on the host plants, creating a mutualism that bolsters the growth of both the bacteria and the plant even in high nutrient scarcity. This is exactly what we have observed in this study. The growth promotion effect of *A. vinelandii* A81 correlates with the positive CFU change (Figure 6), further strengthening the fact that co-existed bacteria have a role in plant growth promotion (Idris et al., 2007; Toyama et al., 2022). Higher CFU and lower CFU values in W. hyalina and *S. polyrhiza* (Fig 3. f) may be due to their relatively larger and smaller surface area per weight, respectively than that of *L. minor*. These results indicate acceptability of wider range environmental bacteria by the host duckweed plants. We found that a soil borne bacterium of *A. vinelandii* A81 can elicit PGP effect on *L. minor* and *W. hyalina*. PGP activity of A81 and P23 on *L. minor* was almost similar in mH medium (Figure 11). SP4 is a PGPB strain for

S. polyrhiza and *L. minor* in the genus *Ensifer* (previously *Sinorhizobium*). SP4 did not form nodule on leguminous plants such as *Canavalia gladiata* and *Phaseolus vulgaris* and no *nod* and *nif* genes are found in its genome (data not shown). It was shown that organic nitrogen is supplied by SP4 and the host *S. polyrhiza* accumulates significantly high amount of Gln upon inoculation of SP4 (Toyama et al., 2022) It remains to be elucidated whether A81 provides inorganic NH_3 immediately after N_2 fixation or organic nitrogen to the host *L. minor*.

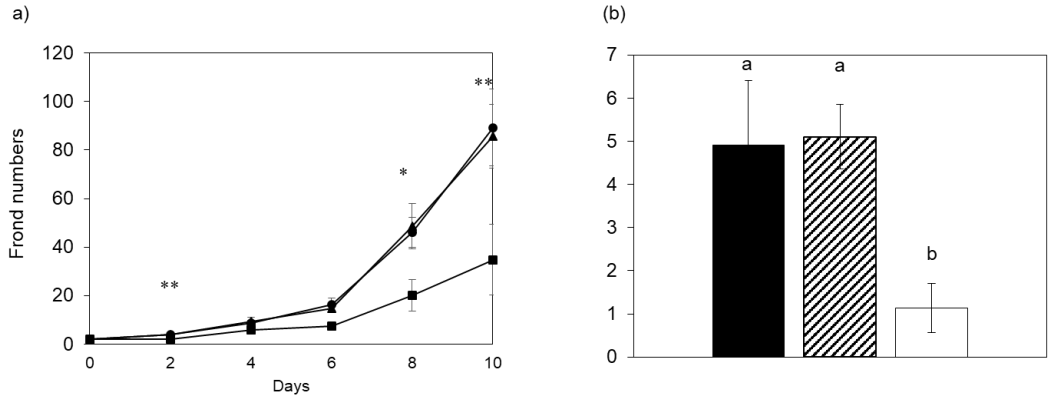


Figure 11. Effect of *A. vinelandii* A81 and *A. calcoaceticus* P23 colonization on the growth of *L. minor* in mH. Symbols are triangle, A81 co-culture; circle, P23 co-culture; square, no-bacteria. (a) Increase in the frond numbers. (b) Dry weight after 10 d culture. All values are mean ± SD (n = 3). Asterisks indicate the significant differences between values with and without bacteria (Student's t-test, * $P < 0.05$, ** $P<0.005$). Different alphabets between treatments indicate significant differences.

It is so interesting that the CFU of non-duckweed originated *A. vinelandii* A81 in this experiment was continuously increased for 30 d in both mH and mH-N medium conditions (Fig. 6), while a natural duckweed originated bacterium P23 showed decrease in CFU on the duckweed to 16% during 10 d co-culture (Yamakawa et al., 2018).This suggests that P23 naturally co-exists with other bacteria to share their niches with an optimal population balance. High stability of mutualism between A81 and *L. minor* may provide us a chance to further develop an effective biomass production technology of *L. minor*. Increase in the CFU at the frond part was evident compared to the root in nitrogen free mH-N

medium condition. This can be reasoned by easy access of A81 to air N_2 in the former area.

We hypothesized that nitrogenase activity of *A. vinelandii* A81 could be a key in the observed PGP effect on *L. minor* under nitrogen free water condition, mH-N medium. In order to verify this hypothesis, we compared the PGP activity of *A. vinelandii* A81 with a nitrogenase negative mutant strain of *A. vinelandii* CA12 in which the nifHDK genes are deleted. It was found that the growth recovery of *L. minor* in mH-N was not observed for CA12 (Figure 5). Attachment PGPB assay (described in 2.2.2.1) was adopted in this experiment where leakage of dead cell lysate containing nitrogen compounds should be minimized. The fact that nitrogenase gene deletion mutant CA12 failed to recover the growth of *L. minor* suggests that direct supply of nitrogen compounds from dead bacterial cells to the plants are negligible level (Fig 4. a-b). *A. vinelandii* has been utilized as a bio fertilizer due to its phosphate solubilization, siderophore production, IAA production, and nitrogen fixation capabilities for terrestrial plants (Aasfar et al., 2021). However, it is early to conclude that these growth promoting factors alone or in concert are responsible for duckweed growth promotion since the condition in the rhizoplane of aquatic plants is largely different from terrestrial plants. It has been reported that exogeneous IAA doesn't affect the growth of *L. minor* (Utami et al., 2018).

Nitrogen fixation is a complex process requiring several key metal ions and an available supply of carbon source (Bellenger et al., 2011; Danapriatna et al., 2013). We initially assumed that the nitrogenase activity, monitored by ARA assay, of A81 would be decreased in the presence of *L. minor*. This could be happened by the following reason. Nitrogen compounds and oxygenic condition resulted from photosynthesis of *L. minor* have a repressing effect on the nitrogenase enzyme activity of the colonized bacteria. However, to our surprise, we have found that A81 cells indeed fixed nitrogen in plant associated condition. There is a report of plant exudates supporting bacterial nitrogen fixation in terrestrial plants (Van Deynze et al., 2018). There is also a recent report that flavone biosynthesis by plants, including Lemnaceae, enhances biofilm formation and nitrogen fixation by diazotrophic bacteria (Pagliuso et al., 2020; Yan et al., 2022). Although in this

study the apigenin and flavonoid derivatives produced by *L. minor* were not measured, the effect of such plant derived secondary metabolites on the nitrogen fixation activity of A81 cannot be ruled out. Furthermore, we observed A81 cells densely colonized on *L. minor* and encapsulated in EPS on the surface of *L. minor* (Figure 12). Significant ability of biofilm formation filled with EPS upon colonization on the *L. minor* may enable the A81 cells to use up oxygen and shape anaerobic condition enabled nitrogen fixation.

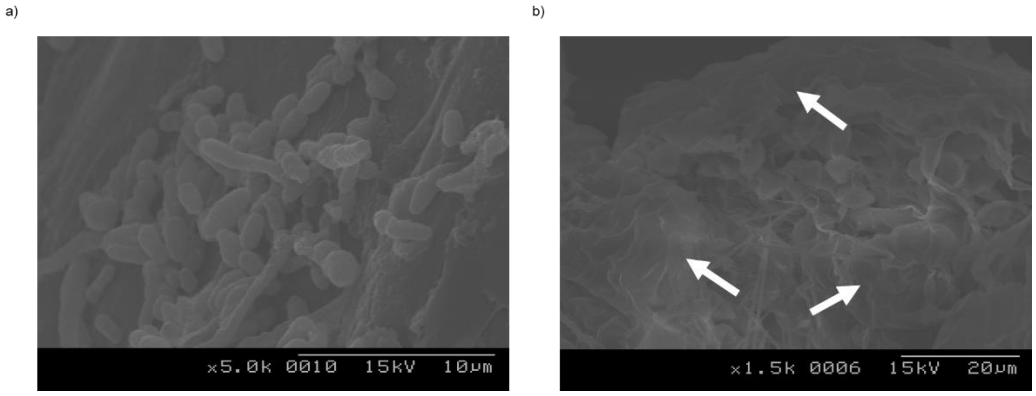


Figure 12. SEM observation of *A. vinelandii* A81 biofilms. A81 cells on polypropylene tube (a) and on *L. minor* (b). A81 cells are encased in significant amount of extracellular polymeric substances upon colonization on the *L. minor*. Arrows indicate bacterial EPS covered cell.

Amount of ethylene produced by *L. minor*/ A81 was clearly higher than not only *L. minor*/ No bacteria but also *L. minor*/ CA12 or *L. minor*/ SP4 (Figure 7). This result can deny a possibility of increased amount ethylene in *L. minor*/A81 due to modification of plant physiology by bacteria.

We further examined the co-culture of *L. minor* with A81 in cell suspension experiment, where the A81 was exposed to a large amount of *L. minor* biomass in nitrogen free BS medium (Figure 8). A pre-incubation time of 6 h was necessary to observe the changes mediated by the plant on the A81 liquid bacterial culture. It was observed that host plant did not reduce the nitrogenase activity but rather prompted the cell proliferation possibly providing bacteria with growth promoting metabolites. Although further studies are needed before we can conclude that *L.*

minor actively bolsters the nitrogenase activity of A81, the results obtained in this study supports reciprocally beneficial symbiotic interaction can successfully constructed between *L. minor* and *Azotobacter*.

2.6. Conclusion

This study demonstrated that the association of soil borne free-living diazotroph *A. vinelandii* A81 showed a growth promoting effect on an aquatic macrophyte *L. minor*. Hence, we have successfully created a novel synthetic duckweed holobiont that would open the gate for biomass production by *L. minor* under severely nitrogen limited water condition. *A. vinelandii* is also an industrially relevant bacterium which is used to produce several compounds such as poly-β-hydroxybutyrate (PHB) (García et al., 2014) and alkyl resorcinol (Funa et al., 2006). Growing *A. vinelandii* cells with valuables production using *L. minor* plant as a scaffold is another challenge for constructing a sustainable industry in the future.

3. *L. minor*/ A81, a potential biological agent for wastewater treatment

3.1. Introduction

Chryseobacterium sp. 27AL, indigenous to the low nitrogen food factory wastewater, successfully promoted the growth of duckweed without competition of nitrogen minerals, *Lemna gibba* (Khairina et al., 2021). On the other hand, *A. calcoaceticus* P23 originated from botanical garden pond water turned to inhibit the duckweed growth in the low nitrogen wastewater condition due to deprivation of limited inorganic nitrogen from the host plant. In this experiment, the potential of the *L. minor*/A81 symbiosis to grow in wastewater condition was explored. A81 promoted the growth of *L. minor* and reduction of excessive Na^+ was observed in the wastewater after the growth of *L. minor/A81*.

3.2. Aim and objectives

i. Application of *L. minor*/A81 symbiosis in wastewater treatment

ii. Elucidation of the performance of the *L. minor*/A81 in nutrient poor wastewater condition and provide a rationale for co-culture experiments in nitrogen poor water

3.3. Method and materials

3.3.1. Industrial wastewater

Wastewater effluent from the final sedimentation tank was collected from a food factory, named hereafter A-WW (Khairina et al., 2021). The color of the A-WW was pale yellow, it was pH 8.4. For long storage in fridge, the wastewater effluent was filter sterilized using filter cup Sartolab 180 C 5, 0.22 μm PES (Sartorius). Filter sterilization was also carried out with the same method before conducting the experiment to prevent contamination. The mineral composition of the wastewater is stated in Table 3 (Khairina et al., 2021). The pH of the A-WW was 8.4, COD was 20mg/L.

3.3.2. Duckweed growth promotion experiments

Duckweed PGPB assay was done in the same method described in 2.2.2.1 with filter sterilized A-WW wastewater instead of mH. Spent A-WW wastewater was analyzed for cation in the ion chromatography by using 10^{-1} diluted samples.

Table 3(a). A-WW mineral analysis data: cations (ND: Not detected)

Cation	Content (mg/L)	Cation	Content (mg/L)
		K	15.2
Al	0.1	Li	0.02
As	0.01	Mg	3.84
B	0.2	Mn	0.01
Ba	0.02	Na	1318
Be	ND	Ni	ND
Bi	0.01	Pb	ND
Ca	10.2	Rb	0.54
Cd	0.25	Se	0.01
Co	ND	Sr	ND
Cr	0.25	V	ND
Cu	0.03	Zn	0.25
Fe	0.05	NH_3	0.21

Table 3(b). A-WW mineral analysis data: anions

Anion	**Content mg/L**

F	0.07
SO_4^{2-}	963.51
Cl^-	189.84
NO_3^-	0.37
PO_4^-	1.45

3.4. Results

3.4.1. Growth recovery in A-WW wastewater

The growth promotion activity was retained when the *A. vinelandii* A81- *L. minor* symbiotic biomass was incubated in A-WW wastewater. After 10 days of incubation, there was a 1.3-folds increase in both frond number and dry weight compared to control in A-WW wastewater (Figure 13. a-b).

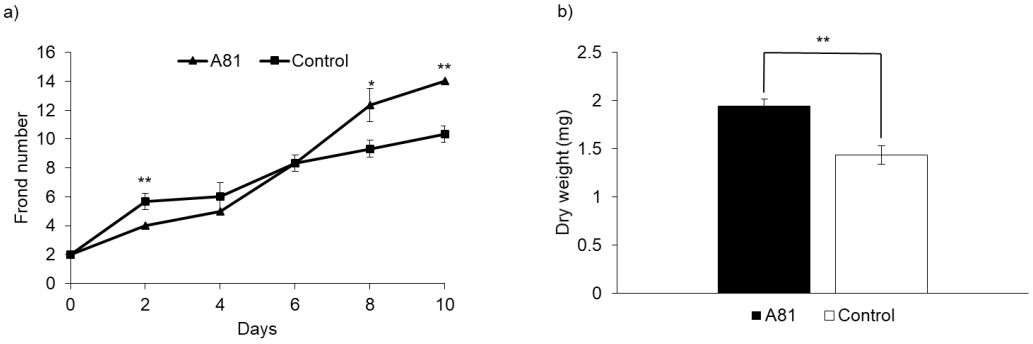


Figure 13. The growth promotion activity was retained when the *A. vinelandii* A81- *L. minor* symbiosis. Effect of *A. vinelandii* A81 attachment on the growth of L. minor (in A-WW, 0.372 ppm) Symbols are closed triangle, *A. vinelandii* A81 co-culture; closed square, no-bacteria. (a) Increase in the frond numbers. (b) Dry weight after 10 d. Increase in frond number of L. minor is shown in bacterial attachment condition in A-WW (AYT wastewater). Symbols are closed triangle, *A. vinelandii* A81 co-culture; closed square, no-bacteria. (a) Increase in the frond numbers. (b) Dry weight after 10 d. *L. minor* showed increased dry weight when grown in association with attached bacterial cells compared to control. All values are mean ± SD (n = 3). Asterisks indicate the significant

differences between values with and without bacteria (Student's t-test, * $P < 0.05$, ** $P<0.005$)

3.4.2.Reduction of cations in spent medium

L. minor/A81 could remove 112% more Na^+ from the medium than *L. minor*/ No bacteria control. All the cations tested were reduced in the spent medium (Table 4). All the cations tested were reduced during the incubation time compared to the control. NH_4^+ concentration below detection limit in spent medium of *L. minor*/A81. Anion concentration of spent medium was not measured.

Table 4. Cation concentration changes after culturing *L. minor*/A81

Cations	A-WW (0 d) ppm	*L. minor*/ A81 (10 d) ppm	*L. minor* /No bacteria Control (10 d) ppm
NH_4^+	0.21	Not detected	Not detected
Na^+	1318	150.41	278.25
Mg^{2+}	3.84	0.88	1.9
Ca^{2+}	10.2	3.2	6.366
K^+	15.2	1.7	3

3.4.3. Change in CFU of colonized A81

Over the time of incubation in A-WW, the CFU of colonized A81 increased slightly from 1.15×10^3/ plant (day 0) to 40×10^3 day (day 10).

3.5. Discussion

There are reports of growth promotion effect by indigenous environmental or wastewater bacteria on *L. minor* (Ishizawa et al., 2017b) , *L. gibba* (Khairina et al., 2021) and *S. polyrhiza* (Toyama et al., 2022), although these bacteria have

never had history to grow with the duckweed. Application of *L. minor* in removing pollutants from wastewater and using wastewater to produce biomass is currently a well-studied sector of sustainability biotechnology. Challenges of growing *L. minor* in wastewater include high salinity, low nitrogen and presence of growth inhibitory organic compounds. In *S. polyrhiza*, salinity ≥ 200mM causes oxidative stress and severely reduces growth (Chang et al., 2012). A-WW had a salinity of 1318 ppm= 48.7 mM. Duckweed *L. minor* may tolerate salt concentrations up to 100 mM before showing significant reduction to chlorophyll content and dry weight. Leaf chlorosis was seen to occur from >50mM NaCl (Liu et al., 2018). The reduction in Na^+ in the presence of A81 can be explained by bio-sorption as reported in various sources (Yang Li et al., 2016; Tewari et al., 2014; Upadhyay et al., 2011) which may have contributed to some of the PGP effects. Moreover, A-WW had nitrogen in the form of NO_3^- (0.372 ppm) and NH_4^+ (0.21 ppm). Duckweed *L. minor* generally having a preference for NH_4^+ (Zhou et al., 2022) quickly absorbed the nitrogen which caused a significant growth rate reduction after the day 6 in absence of A81. On the other hand, in the presence of A81, the growth rate reduction was shown in the beginning which slowly recovered in time possibly due to proliferation of A81 on the *L. minor* and reaching activation barrier (a sufficient number of cells) for nitrogen delivery. The dissolved organic compounds in A-WW did not significantly affect A81 growth. It is generally observed that A81 growing in complex organic L medium has a very slow growth rate compared to mineral BS medium supplemented with sucrose as the sole carbon source.

3.6. Conclusion

A. vinelandii A81 cells improved the growth of *L. minor* both in frond number and dry weight compared to control in wastewater condition. It is not clear in which form A81 provides the nitrogen to *L. minor*. Neither nitrate nor ammonium ions were found in the spent wastewater medium (Table 4), suggesting that the cells were mostly attached to host plant body and planktonic cells were at a negligible level. It also suggests that close association of this manner can avoid the risk of over-dilution of secreted nitrogenous compounds in hydroponic culture.

A81 can produce a significant level of biofilm (Figure 9) and EPS (Figure 12) on *L. minor* frond surface. EPS produced by A81 may possess bio sorption capabilities. Therefore, *L. minor*/A81 symbiosis may be used as a floating, photosynthetic bio sorbent in treating wastewater. This opens a possibility of exploring an EPS or nitrogenase overproducing mutant *A. vinelandii* coupled with duckweed in wastewater treatment and clean biomass production.

4. Factors affecting *A. vinelandii* A81 attachment to *L. minor* and a trial to construct a multiple PGPB consortium

4.1. Introduction

With a view to building a stable duckweed holobiont community, our first attempt at finding a nitrogen fixing symbiont was successful as A81 seems to be a mutually beneficial symbiont for *L. minor* (Shuvro et al., 2022). An important characteristic of a stable holobiont community is conservation of its members. The propagation of *L. minor* mostly being asexual, the stable symbiont may be maintained through many generations of then host plant (Vannier et al., 2018). However, there is a scarcity of knowledge about how a gnotobiotic *L. minor* plant acquires its holobiont community in its aquatic environment. From the bacterial side, involvement of S- layer protein has been reported for the attachment efficiency of *A. vinelandii* (Liew et al., 2015). From plants side that can be number of factors affecting the attachment of bacteria. In aquatic macrophytes, high salinity (Hempel et al., 2008), high planktonic cell concentration (Rimes & Goulder, 1985) and surface roughness (Baker, 2011) correlates with higher bacterial attachment. Nutrient availability affect aquatic macrophyte's ability to promote bacterial growth in suspension condition (Huss & Wehr, 2004) which may also effect bacterial attachment. Importance of bacterial biofilm on the attachment of cells to duckweed was emphasized in recent study (Ishizawa et al., 2020). The biofilm observed in the artificial symbiosis of *L. minor*/A81 (this study) is be capable of acting as a site for cooperation, competition, synchronization and other bacterial interactions (West et al., 2007). Several studies have reported microbial community dynamics of co-inoculation of two bacteria (Ishizawa et al., 2019, 2020; Yamakawa et al., 2018). The PGPBs used in these experiments are previously isolated from duckweed and have a possible history of coevolution with natural duckweed microbial community. The curious question of how an unrelated bacteria will perform with another PGPB in a two bacteria/L. minor co-inoculation system was raised. First, we assessed the factors affecting the attachment of A81 to *L. minor*. Based on that information, the performance of the multiple PGPB consortium was tested. Several well

characterized duckweed PGPB were put to test. The initial trial results showed no significant difference in PGP activity between the co-inoculation compared to their single inoculation counter parts. However, changes in the bacterial CFU of the two co-inoculants suggests a possible competition and commensalism between the members of the community.

4.2. Aim and objectives

i. Optimization of A81 attachment to *L. minor*

ii. Application of A81 as a co-inoculant with known PGPB to build a multiple PGPB consortium

4.3. Method and Materials

4.3.1. Bacterial strains, plant and culture condition

MRB10 (unpublished) were previously isolated from a natural duckweed. 29AL was isolated from wastewater (Khairina et al., 2021). Ps6, A81, SP4 was previously explained in 2.2.1.1. Bacterial culture and storage conditions were same as explained previously.

4.3.2. Determination of factors affecting A81 attachment to *L. minor*

A81 bacterial solutions of 0.3, 0.6 and 3.0 OD_{600} in 50ml NF and mH were prepared in 100 ml conical flasks where >10 aseptic *L. minor* fronds were placed and incubated in plant culture condition for a week. CFU/plant measured after 24h, 48h and 7d.

4.3.3. Duckweed growth promotion experiments

Fresh culture of bacteria (A81, P23, Ps6, MRB10, 29AL) were prepared and centrifuged to retrieve the bacterial cells as pellet. The pellet was then suspended and diluted to make a uniform bacterial cell suspension of 0.3 OD_{600} for single inoculation and 0.15 OD_{600} each for co-inoculation in 50ml mH-N. Two fronds from aseptic duckweed stocks were placed in the medium (n=3). The number of

fronds were counted every 2 days up to 10 days and final CFU colonized on *L. minor* and dry weight of the plants were measured.

4.3.4. CFU measurement

On the 10th day of experiment, 4 fronds/2 roots were randomly selected, rinsed in sterilized MilliQ water two times to remove any planktonic cells. Crushed with biomasher and plated in L, R2A and BS medium. BS medium colonies provide CFU of A81. Other PGPBs were all counted from L plate. A81 and other PGPBs were distinguished by the extremely slow growth of A81 on L plate and colony morphology.

4.4. Result

4.4.1. Determination of factors affecting A81 attachment to *L. minor*

4.4.1.1. 24 hour incubation

NF (nutrient rich) condition exhibited increasing bacterial CFU/plant as the medium cell concentration increased with 0.33×10^4 at 0.3, 0.66×10^4 at 0.6, and 1×10^4 at 3.0 OD_{600}. The mH (nutrient poor) showed a similar trend in the CFU/plant with a reduction of in all treatments (Figure 14. a).

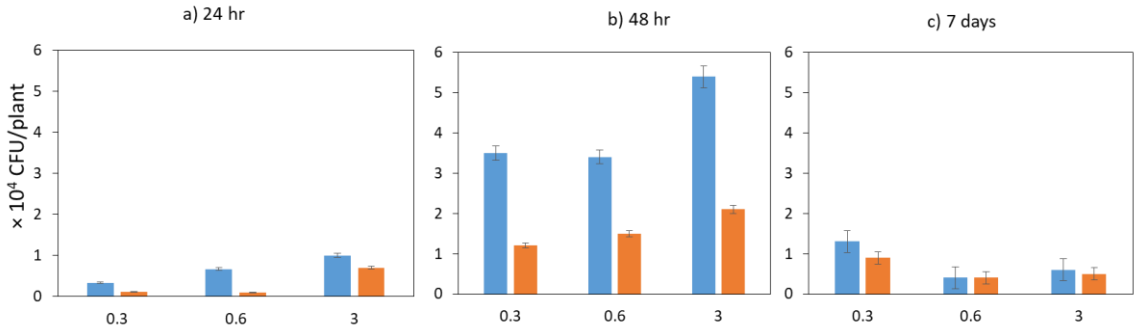


Figure 14. Change in A81 CFU on *L. minor* for 7 days at different amount bacterial suspension. Attached bacterial CFU ($\times 10^4$ CFU/plant) is shown in NF (blue) and mH (orange)

bars at 0.3, 0.6 and 3.0 OD_{600} suspension condition incubated for a) 24 hours, b) 48 hours and c) 7 days. All values are mean ± SD (n = 3).

4.4.1.2. 48 hour incubation

NF condition exhibited a high 5.4× 10^4 CFU/plant at 3.0 OD_{600}. In the other two treatments, 3.5× 10^4 CFU/plant at 0.3 OD_{600} and 3.4 × 10^4 CFU/plant at 0.6 OD_{600} were observed. mH again showed an increasing trend with increasing OD_{600} (1.2× 10^4 CFU/plant at 0.3 OD_{600}, 1.5× 10^4 CFU/plant at OD_{600} and 2.1× 10^4 CFU/plant at OD_{600}) but there was an overall CFU reduction compared to NF treatments (Figure 14. b).

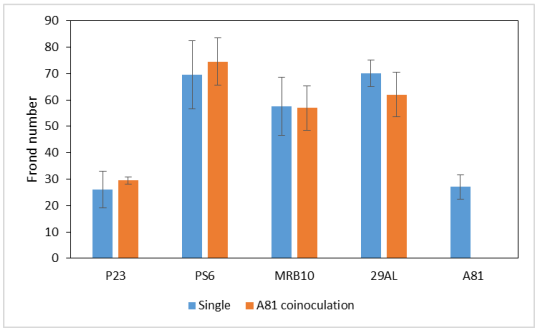
4.4.1.3. 7 day incubation

After 7 day incubation, the attached bacterial CFU/plant was not significantly different in any experimental conditions regardless of the medium nutrient (Figure 14. c).

4.4.2. Duckweed growth promotion experiments

PGP activity was not statistically significant in any of the co-inoculations when comparing to their single inoculation counterparts (Figure 15. a).

a)



b)

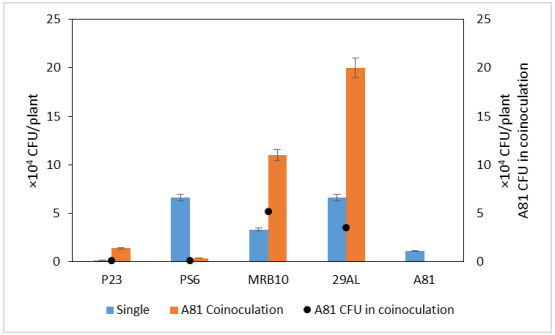


Figure 15. A81 promotes the attachment of P23, MRB10 and 29AL to *L. minor*

(a) Growth promotion activity of PGPB suspension in mH-N in single (blue) and co-inoculation (orange) with A81. Closed bar represent frond number and open circle represent dry weight. All values are mean ± SD (n = 3). (b) $\times 10^4$ CFU/plant of single bacteria inoculation (blue) and co-inoculation (orange) with A81. Closed circle denotes the A81 CFU in the respective co- inoculations.

4.4.3. Bacterial colonization on *L. minor*

Simultaneous increase in the CFU of both members of the co-inoculant was seen in the A81/MRB10 and A81/29AL. A81 CFU increased by 4 and 2.7-folds respectively in A81/MRB10 and A81/29AL compared to single inoculation of A81 (Figure 15. b). MRB10 and 29AL CFU increased 3.3- folds and 3-folds respectively upon co-inoculation with A81 compared to single inoculation. On the other hand, the CFU of A81 decreased in co-inoculation with A81/ P23 and A81/ Ps6. P23 CFU increased slightly compared to the single inoculation. Only Ps6 was seen to be negatively affected by the co-inoculation of A81.

4.5. Discussion

First, factors affecting the attachment of A81 cells to *L. minor* was studied. Availability of more nitrogen in the NF medium compared to mH indeed increased the bacterial CFU/ plant in short incubations up to 48 hours. NF medium is regarded as a nutrient rich medium, where the regular plant metabolic functions prevailed. Enabling the development of a concentration gradient with the continuous accumulation of photosynthetic products in the plant exudates which would in turn promote bacterial chemotaxis to the plant surface. On the other hand, mH is a minimal medium where the *L. minor* exhibits a smaller relative growth rate than NF. The effect of plant nutritional condition on the bacterial proliferation reported previously (Huss & Wehr, 2004) was proved here. The positive trend of increased bacterial CFU/plant with the increasing suspension OD_{600} (Rimes & Goulder, 1985) was also observed. However, in the 7 day incubated samples, cell concentration did not affect the attachment of bacteria. Possibly because of the death of bacterial cells. In the 7 day samples, the effect of medium nitrogen was

also not significant. It can be assumed that the long incubation time (≥7 days) in the bacterial suspension, on the surface of *L. minor*, an equilibrium of selected bacterial communities could have formed. Further experiments are required to assume that this equilibrium may be maintained if all the factors remain constant. Assuming that in this condition, there is a higher amount of A81 biofilm on the plant surface that may facilitate attachment of other bacteria, the multiple PGPB consortium experiment was designed.

In the multiple PGPB consortium experiment, the effect of A81 and co-inoculation of different PGPB on *L. minor* was observed in mH-N condition. mH-N provides a nutrient stress to *L. minor* and it was previously found that A81 proliferation on *L. minor* is higher in mH-N (Figure 6). A nitrogen free medium was chosen to eliminate any positive effect elicited by metabolically affluent plants to the bacteria in the suspension condition (Huss & Wehr, 2004). There was no cumulative growth promotion activity when compared with the single bacterial inoculation and the co-inoculation (Figure 15. a). In previous studies co-inoculation of two different PGPB promoted the growth of *L. minor* by 2.3-folds in attached condition mH medium (Yamakawa et al., 2018). The experimental condition adopted here is different as suspension condition was used to create a stable bacterial community that can coexist at a higher CFU than that of single inoculation. CFU data of the spent medium (day 10) suggests that there was a $>10^3$ fold reduction in the number of cells in suspension of both single and co-inoculation experiments in all of the treatments. Thus, it is probable that planktonic cells were degraded, releasing nutrients in the suspension for the *L. minor* to absorb and act as a nutrient source for the plant.

CFU data of colonized bacteria shows that the bacterial assemblage of the *L. minor* had significantly changed by the addition of A81. Both MRB10 and 29AL CFU on plants had increased with a simultaneously with A81 in co-culture (Figure 15. b) indicating a possible commensalism or a positive interaction on the biofilm covered plant surface. Currently there is not enough data about MRB10, it may be noted that A81 co-inoculation may provide increased attachment of the strain. It was previously reported that in 29AL may colonize up to 4.3×10^3 CFU/plant in

29AL CFU which dropped to 1.1×10^3/ plant in low nitrogen condition (Khairina et al., 2021). Co-inoculation of A81 may increase the CFU of 29AL several folds compared to single inoculation to provide better wastewater treatment efficiency. 29AL was able to utilize organic nitrogen in the cell suspension in order to increase the CFU in single inoculation condition. But in the co-inoculation condition, significant biofilm (Figure 9) and EPS (Figure 12) could have provided a sticky surface for the attachment of bacterial cells. The growth dynamics of Ps6 and P23 have been described before (Yamakawa et al., 2018). In the Ps6 co-inoculation, there might have been competition or antagonism to A81, which led to a significant reduction in the A81 CFU of both bacteria in the co-inoculation experiment. The effect of which can also be seen in the higher frond number of *L. minor* (Figure 15 a) as the lysed cells released more available nutrients in the solution.P23 has shown antagonistic effect against Ps6 (Yamakawa et al., 2018). It is probable that there is an antagonistic effect of A81 towards P23 and Ps6 which is not clear yet.

4.6. Conclusion

Several factors that affect A81 attachment on *L. minor* was elucidated. The preliminary experiment showed there is a potential that A81 may enter in a stable bacterial community on *L. minor* even over long period of incubation time. A81 seems to have a positive interaction with MRB10 and 29AL and a negative interaction with Ps6 and P23 on which is visible on the *L. minor* attachment surface when co-cultured in a tri-partite system. This opens the possibility of studying A81 as an artificial member into an already existing natural duckweed bacterial community. Careful study of the interrelationship between the microbes, further elucidation of this phenomenon in attached bacteria condition and assessment of stability of the consortium is necessary.

5. Differential nitrogen fixation activity of A81 colonized on two types of duckweed

5.1. Introduction

Lemnaceae plants require 0.08 mM/L nitrogen for minimal growth and can tolerate up to 450mM/L depending on the species (Schmid et al., 1988). Nitrogen is one of the limiting factors for producing duckweed biomass. Protein production and duckweed growth is stunted in nutrient poor wastewater or water bodies with suboptimal NO_3^-/NH_4^+ concentrations. Studies on duckweed associated nitrogen fixing bacterial communities is scarce is getting focused. Two PGPB strains from *Pelomonas* sp. were recently isolated from Japanese loosestrife rhizosphere and have shown to possess *nif*H gene. These strains have shown PGP activity in *L. minor*. Several free-living nitrogen fixing microorganisms such as cyanobacteria (Duong & Tiedje, 1985), *Klebsiella* and unclassified aerobic diazotrophs have been reported to be associated with duckweed mats in ponds where they could provide about 15-20% of the duckweed nitrogen requirement through biological nitrogen fixation (Zuberer, 1982). However, the duckweed associated bacterial communities were reported to be devoid of *Azotobacter* sp. which is a dominant group of free-living soil diazotroph. A strain of nitrogen fixing rhizobacterium named *Ensifer* sp. SP4 showed significant growth promotion activity on *S. polyrhiza* (Toyama et al., 2022). ^{15}N radioisotope assay and gene expression analysis showed that nitrogen fixation is not responsible for the duckweed growth promoting effect of the strain and that SP4 does not possess required the nitrogen fixing gene. In our current study, we have found strong evidence that nitrogen fixing strain A81 can promote the growth of *L. minor* but not of *S. polyrhiza*. In a preliminary trial, we also found that the nitrogen fixation activity by the A81 was severely suppressed by the plant in acetylene reduction assay. Hence, further study can bring light into how *S. polyrhiza* and *L. minor* may prefer and maintain functionally different symbiotic partners.

5.2. Aim and objectives

i. Examination of the ability of *A. vinelandii* A81 to form biofilm on the host *L. minor* and *S. polyrhiza*, and measurement of bacterial nitrogen fixation efficiency upon attachment to host plant.

5.2. Materials and methods

5.2.1. Bacterial strains, plant and culture conditions

Azotobacter vinelandii A81 and *Ensifer* sp. SP4 and their respective culture conditions have been described in 2.2.1.1 and 2.2.1.2. *Lemna minor* and *Spirodela polyrhiza* was described in 2.2.1.3.

5.2.2. Nitrogen fixing activity

Nitrogen fixing activity of bacteria colonized on *L. minor* and *S. polyrhiza* was measured and quantified by the acetylene reduction assay explained in 2.2.3.5.1.

5.2.3. Fluorescence microscopy

Fresh culture of A81 was prepared by incubating 50ml BS medium at 30 °C for 2 days starting from 0.01 OD_{600} on day 0. Bacterial culture was centrifuged at 7,700 × g for 10 min at 4°C to retrieve the bacterial cells as pellet. The pellet was washed with mH medium, re-suspended, and diluted to make a uniform bacterial cell suspension of 0.3 OD_{600} (about one million cells) in 50 ml mH medium, where two aseptic plant bodies (two fronds with two roots) *L. minor* and *S. polyrhiza* were placed in individual plant culture dishes and co-cultured under plant growth condition. After 2 days of co-culture, the plants were taken out, rinsed with sterile MiliQ water and stained with either LIVE/DEAD Baclight bacterial viability assay kit or Calcofluor white and subjected to fluorescence microscopy. Control plants without bacterial attachment were also prepared in the same method. Stained bacterial cells/biofilm on duckweed roots were observed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

5.2.3.1. LIVE/DEAD Baclight bacterial viability assay

Bacteria colonized duckweed was stained with the LIVE/DEAD Baclight bacterial viability kit L13152 (Molecular Probes-Invitrogen, Sandiego, CA) that uses SYTO 9 and propidium iodide. SYTO 9 is a nucleic acid stains that can permeate cells and stains the nucleic acid e.g., DNA regardless of the cell membrane integrity. On the other hand, propidium iodide can only stain the nucleic acids of membrane compromised cells. Using these two dyes simultaneously, live and dead bacterial cells can be visualized. Live/ viable cells fluoresce green while the non-viable/ dead bacterial cells fluoresce red (Ou et al., 2019; Buysschaert et al., 2016). LIVE/DEAD BacLight staining reagent 1X stock solution was prepared by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a 10 mL sterilized MiliQ water in separate 15ml falcon tubes. Bacteria colonized duckweed was submerged in 500 μL of 50:50 solution made from 1X SYTO 9 and 1X propidium iodide stock solutions in a 1.5-mL micro centrifuge tube and kept in dark for 15 minutes before observing with the fluorescence microscope.

5.2.3.2. Calcofluor white

Bacterial biofilm often contains polysaccharides such as β-glucan and cellulose. Calcofluor white is a non-specific dye which can bind to these β-linked polysaccharides (Maeda et al., 1967). Upon strong attachment to the polysaccharides present in the biofilm, the dye can be visualized by UV light or blue light. Presence of intense fluorescent bright green to blue indicates presence of bacterial biofilm whereas the plant cellulose shows a uniform fluorescence of the cell walls. In this experiment, bacteria colonized duckweed roots were put on a clean glass slide where one drop of Calclofluor white stain and one drop of 10% KOH solution was added. Coverslip was placed and the specimen was examined under blue light after one minute.

5.3. Results

A81 cells showed a variation in ARA activity while attached on two types of duckweed. On *L. minor*, A81 showed 154.96 ethylene (µmole)/g fresh weight with an initial CFU 0.023×10^6/g fresh on the other hand, in association with *S. polyrhiza*, A81 showed 44.99 ethylene (µmole)/g fresh weight with an initial CFU 0.77×10^6/g fresh. Post assay CFU had a 20-folds increase in the *L. minor* and only 1.85-folds increase in *S. polyrhiza*. (Figure 16). The student's t-test has shown the difference is statistically significant. SP4 was used as a negative control for the nitrogen fixation experiments. Live bacterial cells were observed in roots of both species but *L. minor* (Figure 17. a) showed higher fluorescence in its roots than *S. polyrhiza*. Biofilm formation by the bacteria was observed in *L. minor* roots (Figure 17 c) which was not observed in the *S. polyrhiza* root (Figure 17 d).

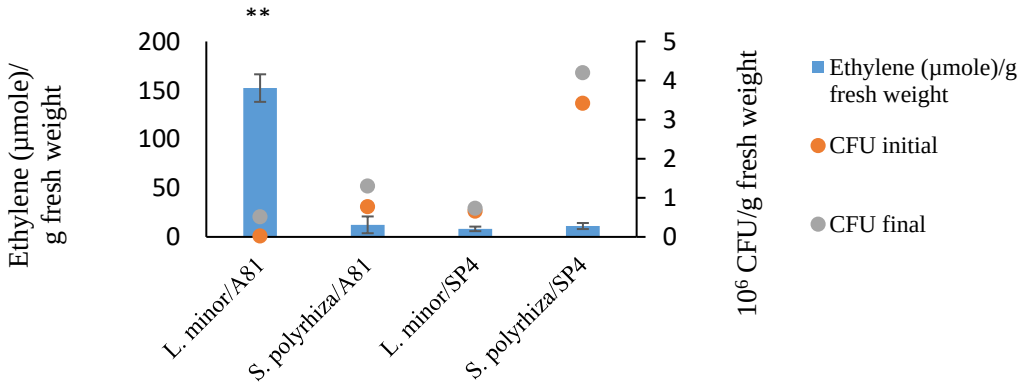


Figure 16. Differential nitrogen fixation activity of A81 colonized on two types of duckweed. Blue bars represent ethylene µmole/g fresh weight *L. minor*. All values are mean ± SD (n = 3). Asterisks indicate the significant differences between values (Student's t-test, * P < 0.05, ** P<0.005).

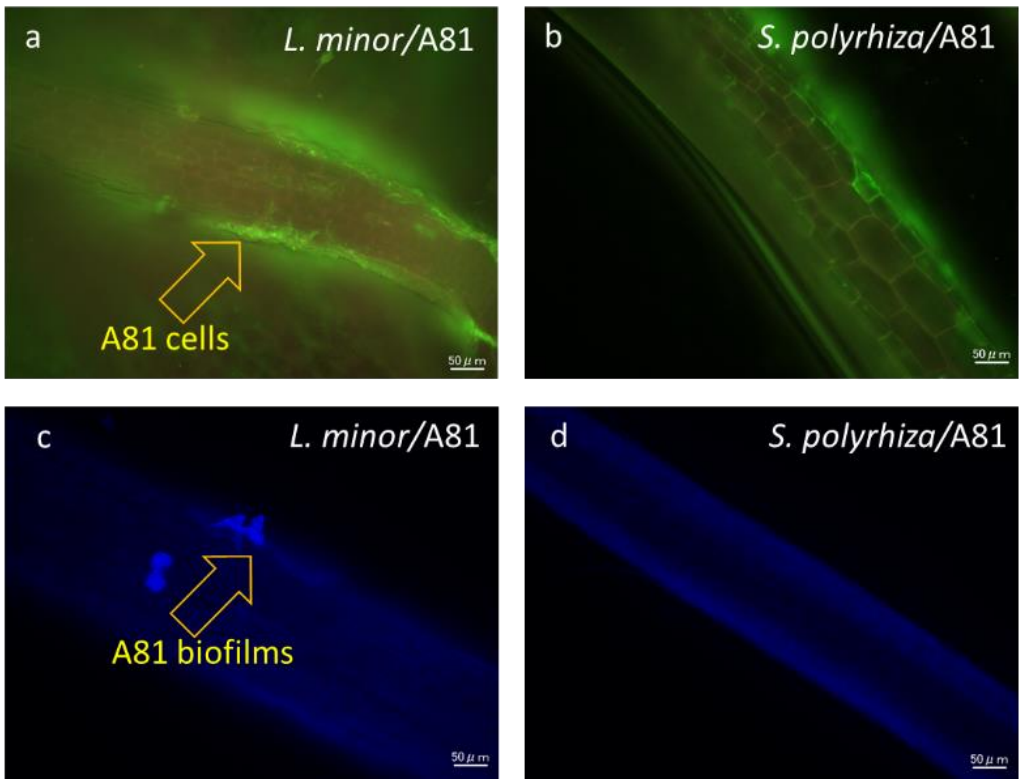


Figure 17. Fluorescence microscopy. Figure a, b: Live/Dead Baclight bacterial viability assay; SYTO 9 and propidium iodide stained roots of *L. minor* and *S. polyrhiza* respectively. Strong green fluorescence indicating live bacteria. Figure c, d: Calcofluor White stained roots of *L. minor* and *S. polyrhiza* respectively. Strong blue fluorescence is indicative of bacterial biofilm.

5.4. Discussion

Increase of CFU over assay period is indicative of the viability of the cells. In the case of *L. minor*, the CFU increase was 20-folds higher, which can be correlated with the very high ARA activity. In the case of *S. polyrhiza*, both CFU increase, and ARA was significantly low. ARA activity of A81 was hypothesized to stimulated by flavonoid biosynthesis that also occurs within duckweed *Lemna* and *Spirodela* (Pagliuso et al., 2020; Yan et al., 2022). In *Spirodela* the flavonoid compounds are reported to be at a higher concentration. A higher flavonoid should have prompted a higher ARA by the A81. But we have observed a contradictory result. It is probable that *S. polyrhiza* root exudates may not be able to secrete

flavin and apigenin compounds although it is produced inside the plant. On the other hand, *L. minor* root exudates may be able to excrete such compounds that can eventually prompt the bacterial nitrogenase activity.

The annotated genome of *L. minor* is 481Mb while *S. polyrhiza* genome is only 150Mb (An et al., 2018) indicating more diverse metabolic capabilities of *L. minor*. There is evidence for *L. minor* being a more efficient in removing NH_4^+ in comparison to *S. polyrhiza*. Genome analysis has revealed that the genes encoding GS (Glutamine synthetase) and GOGAT (glutamine oxoglutarate aminotransferase) expanded to 12 and 21 members in *L. minor* as opposed to only 7 and 11 respectively in *S. polyrhiza* (An et al., 2018). A faster absorption of NH_4^+ may relieve the suppressing effect on nitrogenase in the rhizosphere, elevating nitrogenase activity of the A81 whereas the A81 attached to *S. polyrhiza* may have been metabolically inactive due to excessive NH_4^+. It is hypothesized that change in oxygen in the reaction vial due to different rate of photosynthesis of the duckweed may have an effect on the nitrogenase activity. Nitrogenase can be irreversibly inactivated by oxygen (Rubio & Ludden, 2008). However, significant EPS and biofilm should provide protection from oxygen (D. Wang et al., 2017). This data supports the hypothesis that *Ensifer* sp. SP4 strain doesn't provide any PGP effect from fixing nitrogen on *S. polyrhiza* (Toyama et al., 2022) and indicates a close association between SP4- *S. polyrhiza* is prevalent as found in nature. On the other hand, in nature than A81- *S. polyrhiza*.

5.5. Conclusion

Being aquatic macrophytes neither *L. minor* nor *S. polyrhiza* was found to possess any reported association with *A. vinelandii.* However, a differential behavior of PGP activity (Figure 4) and nitrogen fixation was proved (Figure 16). It is found that *A. vinelandii* A81 is capable of fixing nitrogen only when attached with *L. minor* but is unable to fix nitrogen when attached with *S. polyrhiza*. This is further supported by the detrimental effect of A81 on *S. polyrhiza* dry weight (Figure 4. e) and a low CFU increase (Figure 4. f) when co-cultured for 10 d. Further study into this phenomenon can help us understand how these two types of duckweed

interact with nitrogen fixing bacteria in their habitat and open the door to further exploiting biological nitrogen fixation for growing valuable duckweed biomass.

6. Examination of extracellular polymeric substances from *A. vinelandii* as a plant growth promoting factor

6.1. Introduction

It is generally assumed that colonized bacterial cells may provide growth promotion effect on *L. minor* through a variety of mechanisms. In order to elucidate the bacterial PGP mechanisms of *Azotobacter vinelandii* on *L. minor*, we initially focused on A81 strain and elucidated the nitrogen fixation related PGP effect in nitrogen free medium. However, in nitrogen containing medium, the PGP effect of A81, CA and CA12 did not show significant difference (Appendix figure S1); Leading to a hypothesis that the CA12 strain or its parent strain named CA (*A. vinelandii* ATCC 13705), may utilize a different PGP mechanisms independent of the nitrogen fixing ability. We then began hypothesizing if the PGP effect of different strains correlate with nitrogen fixing rate. Upon investigation, we have found that A81 strain has a higher nitrogen fixation rate than that of CA (Appendix figure S2- S3). The higher frond number and dry weight also support the hypothesis that PGP effect of *A. vinelandii* is correlated with the nitrogen fixation rate of the strain in nitrogen free medium (Appendix figure S1). But in nitrogen containing medium, the PGP effect must be independent of nitrogen fixation rate of the strains. Hence, we started the investigation on PGP mechanism other than nitrogen fixation. Preliminarily, A81 and CA showed huge differences in biofilm production (Figure 9) and mucosity on the agar plate (data not shown). *Azotobacter* bacteria is known to produce a large amount of alginate which is one of extracellular polymeric substances, EPS (Sabra et al., 2001). EPS is generally composed of exopolysaccharides, extracellular DNA, and extracellular proteins, and often function as matrix upon forming biofilms by bacteria (Flemming et al., 2007). The *A. vinelandii* A81 biofilm was indeed encased in EPS upon forming biofilms on surface of a polypropylene tube (Figure 12). We wondered if the EPS from A81 is one of plant growth promoting (PGP) factors. EPS from *A. vinelandii* CA was also prepared as reference sample. EPS, from *Azotobacter vinelandii* was isolated, analyzed, purified and subjected to PGF assay to identify any PGP effect.

6.2. Aim and objectives

i. Purification, characterization and assessment of specific PGP compounds extracted from bacterial culture supernatant.

ii. Analysis of functional, chemical, structural differences between the EPS produced by A81 and CA strains.

iii. Identification of genomic differences between the two strains.

6.3. Method and materials

6.3.1. Bacterial strains and culture conditions

A81, CA12 strains were explained in Chapter 2.2. The parent strain of CA12, *A. vinelandii* ATCC 13705 hereby denoted as CA was collected from ATCC. Bacterial and plant culture condition were described in Chapter 2.2.

6.3.2. Alginate production profiling

Fifty ml BS medium (supplemented with 2.25g/L CH_3COONH_4 as nitrogen source) was inoculated with bacterial pre culture (A81, CA12) to make 0.01 OD in 100 ml conical flask. The flasks were incubated at 30°C for 120 h and every 24 h, 0.5 ml sample was taken and centrifuged at 4°C at 7,700 × g. The culture supernatant was analyzed for secreted alginate in a method described in 4.2.6.3.

6.3.3. Preparation of crude EPS

EPS was extracted with a similar but modified protocol for precipitation of DNA (Green & Sambrook, 2016). In brief, bacteria were pre-cultured in nitrogen free 50 ml BS medium for 72 h. CA 12 had 2.25g/L CH_3COONH_4 as nitrogen source. It was found that addition of nitrogen in the bacterial medium does not have any influence on the quality (PGP effect on *L. minor*) or yield of the EPS. One L BS medium was inoculated with 1% with the pre-culture and incubated for 120 h before spinning down the cells at 31,304× g for 30 minutes at 4°C. It was found that if the at incubation time was >120 hours, protein content of the supernatant started to increase possibly due to the lysis of cells. Collected supernatant was filter sterilized with 0.2 µm Millipore filter to remove bacterial cells and mixed with double volume of ethanol and stored in 4°C for 48 hours. The mixture was

then centrifuged at 48,912 × g again for 30 minutes and pellet was dissolved in MilliQ water and freeze dried and used for further analysis. EPS derived from A81 and CA will be hereon denoted as EPS_{A81} and EPS_{CA} respectively. Yield of EPS_{A81} and EPS_{CA} was 357.14 mg/L and 444.28 mg/L respectively.

6.3.4. EPS_{CA} solubilization

The direct application of 100 μ g/ml EPS_{A81} (79% w/w alginate) or alginic acid sodium salt from brown alga (Sigma- Aldrich, 180947) did not show significant PGP effect on *L. minor*. On the other hand, EPS_{CA} showed significant PGP effect. Hence, purification and further analysis was done for only EPS_{CA}. The crude EPS_{CA} was not soluble in MilliQ water at >10µg/ml concentration. Hence, a suitable pretreatment method was explored. In order to confirm if the undissolved EPS_{CA} is of any importance as a PGP compound, two treatment methods were tested. First treatment "dissolved" was done by adding NaOH to the 50µg/ml EPS_{CA} solution to pH 9 and then returning the pH to 7 with HCl which yielded a clear solution. The second treatment "filtered" was done by filtration with 0.2 µm Millipore filter to remove any undissolved sediments from the solution. Both the treatments were tested using plant growth promoting factor (PGF) assay explained in 4.2.9. Since no significant difference between "dissolved" and "filtered" was found (Figure 18) in terms of growth promotion effect in *L. minor*. All treatments from here on is done with "dissolved" EPS.

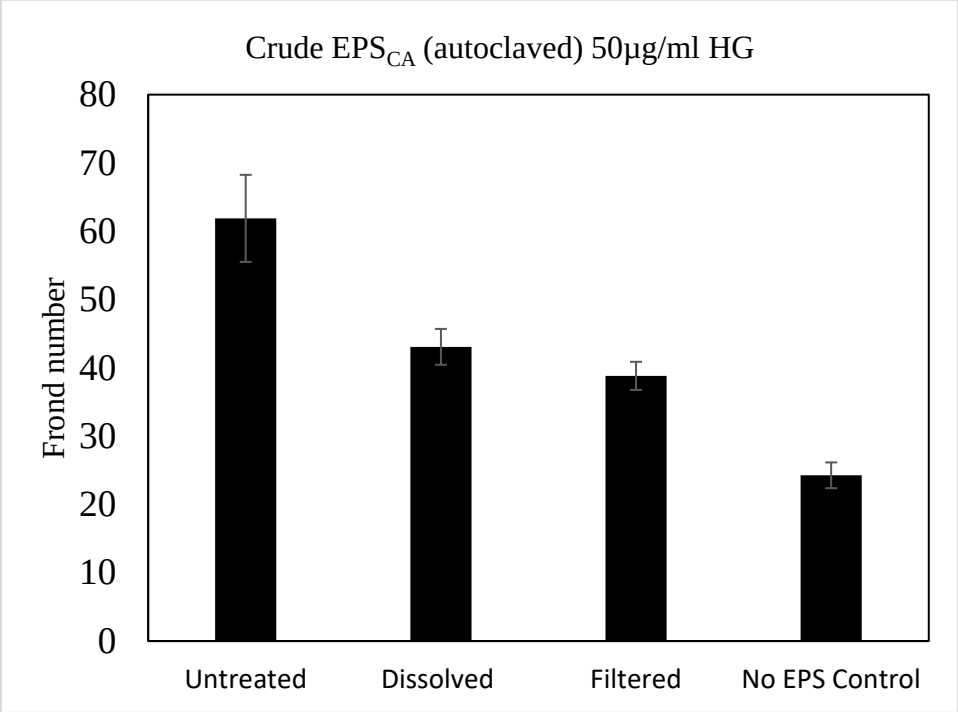


Figure 18. Effect of various pretreatments on the PGP function of EPS_{CA}. All values are mean ± SD (n = 3)

6.3.5. Purification of EPS_{CA}

6.3.5.1. Phenol treatment to eliminate protein fraction

250 mg EPS_{CA} was dissolved in 250 ml MilliQ water to make a solution (1mg/ml) in 500 ml conical flask where 250 ml of 90% phenol was added and incubated at 60°C for 20 minutes with vigorous shaking every 2 minutes. The mixture was then centrifuged at 7500 RPM at 15°C for 15 min and supernatant collected. Same volume of MilliQ water was added to the pellet and incubated again at 60°C for 20 minutes with vigorous shaking every 2 minutes before centrifugation. The supernatant was collected and combined with the previously collected supernatant. The process was repeated one more time to remove residual protein contamination.

6.3.5.2. Dialysis to eliminate salts

SpectraPor cellulose dialysis tube (SpectraPor 3 dialysis membrane, 132724, MWCO: 3.5kD) was pre-treated using 0.02% $NaSO_4$ for 40 minutes before inserting sample liquid into the tube. Then both ends were clamped shut and

dialyzed for at least 48 hours in deionized water until no detectable peak at 280 nm wavelength in the spectrophotometer analysis which indicate protein or phenol contamination. The liquid was freeze dried and used for next step.

6.3.5.3. DNase treatment to eliminate DNA fraction

250 mg EPS was dissolved in a 500 ml DNase buffer solution consisting of 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (pH 7.5) in a 500 ml conical flask where DNase I from Bovine (Sigma Aldrich) was added at 10 µg/ml concentration and incubated statically at 37°C for 1h. EPS was extracted from this liquid using the ethanol precipitation method described above and freeze dried. No peak at 260 nm or 280 nm in the UV-VIS spectrum indicated the purity of the sample.

6.3.6. EPS characterization

6.3.6.1. Protein

EPS protein content was measured by Bio-Rad assay kit (Catalog #500-0006) with a BSA standard curve.

6.3.6.2. Total Sugar

Sugar content of EPS was measured by adding 50µL 90% phenol and 2ml H_2SO_4 to 1mg/ml EPS sample solution. After 10 minute incubation, OD 490 was measured and monomeric sugar was quantified using a standard curve made with glucose and sodium alginate (M. Dubois et al., 1951; Michel Dubois et al., 1956).

6.3.6.3. Alginate assay and total uronic acid

EPS alginate content was measured by alginate assay (Knutson & Jeanes, 1968; Yoneyama et al., 2015) that specifically quantifies the uronic acid moiety. Briefly, in a glass test-tube 87.5 µL 1mg/ml sample was added to 732.5 µL ice chilled H_2SO_4, where 17.5 µL 45mM Boric acid solution and 25 µL 0.1% w/v carbazole was added immediately and incubated for 30 min before measuring OD_{530}. The alginate was quantified using standard curve made with different concentrations of Alginic acid sodium salt.

6.3.6.4. Quantification of alginate using phenol- sulfuric acid method

Monomers of alginate were measured using the total sugar measuring method (M. Dubois et al., 1951; Michel Dubois et al., 1956) using a standard curve made with different concentrations of Na- alginate.

6.3.6.5. Sugar analysis by HPLC

Monomeric sugar analysis was performed by High Performance Liquid Chromatography (HPLC). Powdered sample of 1.01 mg purified EPS_{A81} and EPS_{CA} was incubated with 100µL 4 M Trifluoroacetic acid at 100℃ for 3hours. Then analyzed with the following HPLC protocol: Device: ACQUITY Arc (Waters), Column : PN-PAK C18 (3.0 x 75 mm), Solvent : Borate buffer／Acetonitrile, Flow rate : 0.5 mL / min, Detection : UV (Ex: 305nm , Em: 360nm). HPLC was conducted by Proteinova.

6.3.6.6. FTIR- ATR analysis

EPS of the two strains of *A. vinelandii* was subjected to analysis with Fourier-Transform Infrared Spectroscopy- Attenuated Total Reflection (FTIR-ATR). This widely used carbohydrate characterization technique is based on the principle that infra-red light changes the dipole moments in molecules. A radiation is emitted when IR light excites the chemical bonds. As infrared light (photon) is absorbed by a molecule, bonds within the molecule reaches different excitation state and this creates vibration or rotations such as symmetric or asymmetric stretching, twisting, wagging etcetera. The vibrational energy released from corresponding bonds are consistent and correlate to different functional groups in a molecule. Thus, a careful study of the peaks in FTIR-ATR can provide an understanding about both the structure and the functional groups of a molecule. This method is widely used to study and characterize carbohydrates present in bacterial EPS (Rehman et al., 2021) and seaweed polysaccharides (Pacheco et al., 2021). For this experiment, dried and milled sample of 1.0 mg was used. FTIR-ATR spectra were recorded on a JASCO FT/IR 6100 crystal structure analyzer (Japan) from

550 to 4000 cm^{-1} with 128 scans, each at a resolution of 2 cm^{-1}. Data was analyzed and recorded using Spectral manager software provided by JASCO. Peaks were then identified using Spectragryph 1.2.16.1 developed by Dr. Friedrich Menges.

6.3.7. Alginate lyase activity measurement (qualitative method)

Alginate lyase activity was qualitatively measured by the following method. Overnight bacterial pre- cultures were used to inoculate 5 ml liquid L medium supplemented with 0.2% Sodium alginate (Sigma Aldrich) and incubated for 48 hours at 30°C. 0.5 ml bacterial culture was centrifuged at 7,700 × g, 4°C for 10 minutes. 0.2 ml supernatant was taken in a glass vial and 2 ml acidic albumin solution (Kitamikado et al., 1990) was added. A clear solution indicates significant alginate lyase activity while turbid solution indicates negligible or no alginate lyase activity.

6.3.8.Preparation of alginate lyase treated EPS_{A81}

Sample was prepared according to a protocol for alginate oligosaccharides preparation (Z. Zhang et al., 2004) with slight modification. 5g/L EPS solution was prepared by dissolving 250mg EPS into 50ml 50mM Trish- HCL (pH 7.5) buffer supplemented with 0.1 M NaCl where 50 units of alginate lyase enzyme from *Flavobacterium* sp (Nippon gene, Code No. 319-08261) were added. The reaction was carried out at 30°C for 24 h. The next steps were done according to method described in (Cao et al., 2007). The solution was heated at 100°C for 10 minutes in order to stop the reaction and equal volume of ethanol was added and centrifuged at 7,700 × g for removal of intact polysaccharide form alginate. The supernatant was filtered with 0.45 µm Millipore filter, and mildly heated (<100°C) while shaking to facilitate ethanol evaporation. After sufficient removal of ethanol, the alginate lyase treated EPS, hereafter alt-EPS was freeze dried and stored at -20°C until used for PGF assay.

6.3.9.Plant growth promoting (PGP) assay

6.3.9.1. PGP assay comparing crude EPS

Aseptic *L. minor* was put in 10 ml autoclaved mH supplemented with 100 µg/ml crude EPS, Yeast mannan (Sigma Aldrich), Na- alginate (Sigma Aldrich) in 6 well plates (n= 3) and incubated for 10 days before measuring final frond number and dry weight. The experiment was repeated in the same method using 50ml mH supplemented with 1, 10, 50, 100 µg/ml crude EPS_{CA}. Frond number and dry weight was measured after 10 day incubation.

6.3.9.2. PGP assay of pure EPS_{CA}

PGP effect of EPS was reconfirmed by placing 2 fronds of aseptic *L. minor* in 10 ml autoclaved mH supplemented with 0, 10, 50 µg/ml purified EPS in 6 well plates (n=3). Dry weight, fronds number measured after 10 day incubation.

6.3.9.3. PGP assay of alt-EPS_{A81}

PGP activity of alginate lyase treated EPS_{A81} hereby denoted as alt-$EPS_{A81}$was measured by placing 2 fronds of aseptic *L. minor* in 10 ml autoclaved mH supplemented with 100 µg/ml alt-EPS_{A81} in 6 well plates (n=3). Dry weight, fronds number measured after 10 day incubation.

6.3.10. Genomic data comparison between A81 and CA

6.3.10.1. Comparison of alginate biosynthesis genes in NCBI database:

Whole genome data of *A. vinelandii* NBRC 13581 RefSeq sequence NZ_BCTD01000001.1 was compared with *A. vinelandii* CA Genebank sequence CP005094.1= RefSeq sequence NC_021149.1 found in NCBI database manually and also using BLAST. Alginate biosynthesis genes were obtained from (Pacheco-Leyva et al., 2016).

6.3.10.2. Analysis of genomic data using JGI IMG/ M

Genome analysis was also conducted using Integrated microbial genome and microbiomes (JGI IMG/M) website (Chen et al., 2022; Mukharjee et al., 2022). CA had a fully finished sequence on the other hand a finished sequence was not found for A81. Hence, the analysis was performed with the permanent draft of A81 available in the database. The permanent draft of A81 was short on 275 genes compared to CA finished sequence (Figure S10). Genomic analysis was divided in the following steps:

i. Average nucleotide identity (ANI): Provided the relatedness between the two sequences.
ii. Phylogenetic Profilers: In many cases the differences in physiology, phenotypic properties and ecology of different organisms can be attributed to the differences in their gene content, i.e., the differences in abundance of various gene families, including the ultimate case of certain genes being present in one genome but not in another genome(s) and vice versa. Phylogenetic profiler provides an understanding of genes unique to or homologous across compared genomes.
iii. Abundance profile of overall genes: Identification of genes uniquely present/absent in the sequence of A81 and CA.
iv. Glucosyltransferase gene abundance comparison: Provides a comparison of genes closely related to EPS production found in the sequence.

6.4. Results

6.4.1. Alginate production profiling

A81 showed a significantly higher alginate production in the supernatant that increased with time during the course of the measurement. The alginate production activity was 187.6 µg alginate/ OD_{600} at 96 hours. On the other hand, the highest alginate production by CA12 was 15.13 µg/ OD_{600} at 48 hour which gradually decreased to 1.8 µg per OD_{600} at 96 hours (Figure 19). An alternative

graph showing alginate produced (μ g/ml) in the culture medium and change of bacterial OD_{600} with time is provided in the appendix (figure S11).

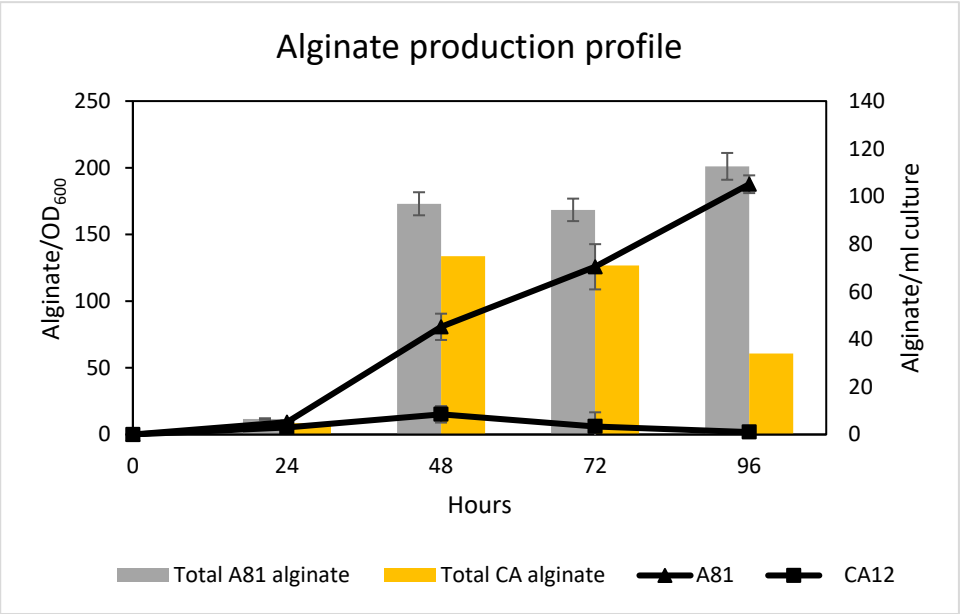


Figure 19. Alginate production over time in the bacterial supernatant of *A. vinelandii.* Strains A81, closed triangle; CA12, square. Bars represent total alginate in 1ml bacterial liquid culture. Grey bar, A81; Yellow bar, CA12. All values are mean ± SD (n = 3).

6.4.2. EPS protein, sugar and alginate content

6.4.2.1. Protein

Both the crude EPS_{A81} and EPS_{CA} contained small fraction of protein contamination 23.9± 0.38 µg/mg and 22± 0.52 µg/mg respectively. After phenol treatment, purified EPS_{CA} had undetectable level of protein (Table 5).

6.4.2.2. Total Sugar

The total sugar of EPS_{A81} and EPS_{CA} was 224.3±3.55 µg/mg and 120.25±1.5 µg/mg respectively quantified by phenol-sulfuric acid measurement using Glucose standard curve. Although the method (Dubois et al., 1951) is sensitive for all monomeric sugars, the highly reduced number in the sugar concentration maybe caused by some contaminant in the crude extract that interfered with the

colorimetric assay. The total monomeric sugar concentration was 570.33±2.3 µg/mg for the purified EPS_{CA} (Table 5).

6.4.2.3. Alginate assay and total uronic acid

Crude EPS_{A81} showed a significantly high 795.27± 5.15 µg alginate. On the other hand, the EPS_{CA} had 31.35±2.46 µg/mg which negligibly changed after the purification process (Table 5). There is a discrepancy in the amount of alginate measured when Phenol-sulfuric acid method was used. The Phenol- sulfuric acid method is used for measuring total sugar content of a sample and usually not used with alginate standard curve for quantification. The high amount of alginate (245.61 μ g/mg) of EPS_{CA} using phenol sulfuric acid and Na-alginate standard possibly shows some non-specific binding and can be considered as an error in the method. Hence, the value of alginate quantified from the boric acid- carbazol-sulfuric acid with Na-alginate standard was chosen as the viable data.

Table 5. Estimation of EPS composition using different methods

EPS	Alginate µg/mg (Phenol-Sulfuric acid) Na-Alginate standard	Alginate µg/mg (Boric acid-Carbazol-Sulfuric acid) Na- alginate standard	Total sugar µg/mg (Phenol-Sulfuric acid) Glucose standard	Protein µg/mg (BSA Standard)
Crude EPS_{A81}	824.56±19.75	795.27±5.15	224.3±3.55	23.9± 0.38
Crude EPS_{CA}	245.61±8.45	31.35±2.46	120.25±1.5	22± 0.52
Purified EPS_{CA}	Not done	33.446±7.9	570.33±2.3	Not Detected

6.4.2.4. Sugar composition analysis

EPS_{CA} had a 3.6-folds higher molar content of Mannose and 3.3-folds lower Rhamnose content compared to EPS_{A81} per mg sample. The molar ratio of Gal: Glc: Ara: Rib of EPS_{CA} and EPS_{A81} was 0.078: 1.0: 0.04: 0.08 and 0.04:1.0: 0.03: 0.11 respectively. Xylose, Fucose, N- Acetyle mannosamine were only found in EPS_{CA} and were not detected in EPS_{A81} (Table 6 a, b). A complete report on the sugar analysis is provided in the appendix (Figure S6-S8).

6.4.2.5. FTIR-ATR analysis

FTIR-ATR analysis showed distinct peak at 1204 cm^{-1} for EPS_{CA}. Similar peak and shoulder locations have been reported for sulfate esters in available literatures (Lloyd et al., 1959; Pacheco et al., 2021). At similar threshold and prominence, EPS_{CA} did not show any peaks at higher wavenumber after 1204 cm^{-1}. EPS_{A81} resembled the Na- alginate peaks at 668.8, 1418, 1507, 1559 cm^{-1} peaks (Figure 20). The complete spectra is provided in the appendix (Figure S9).

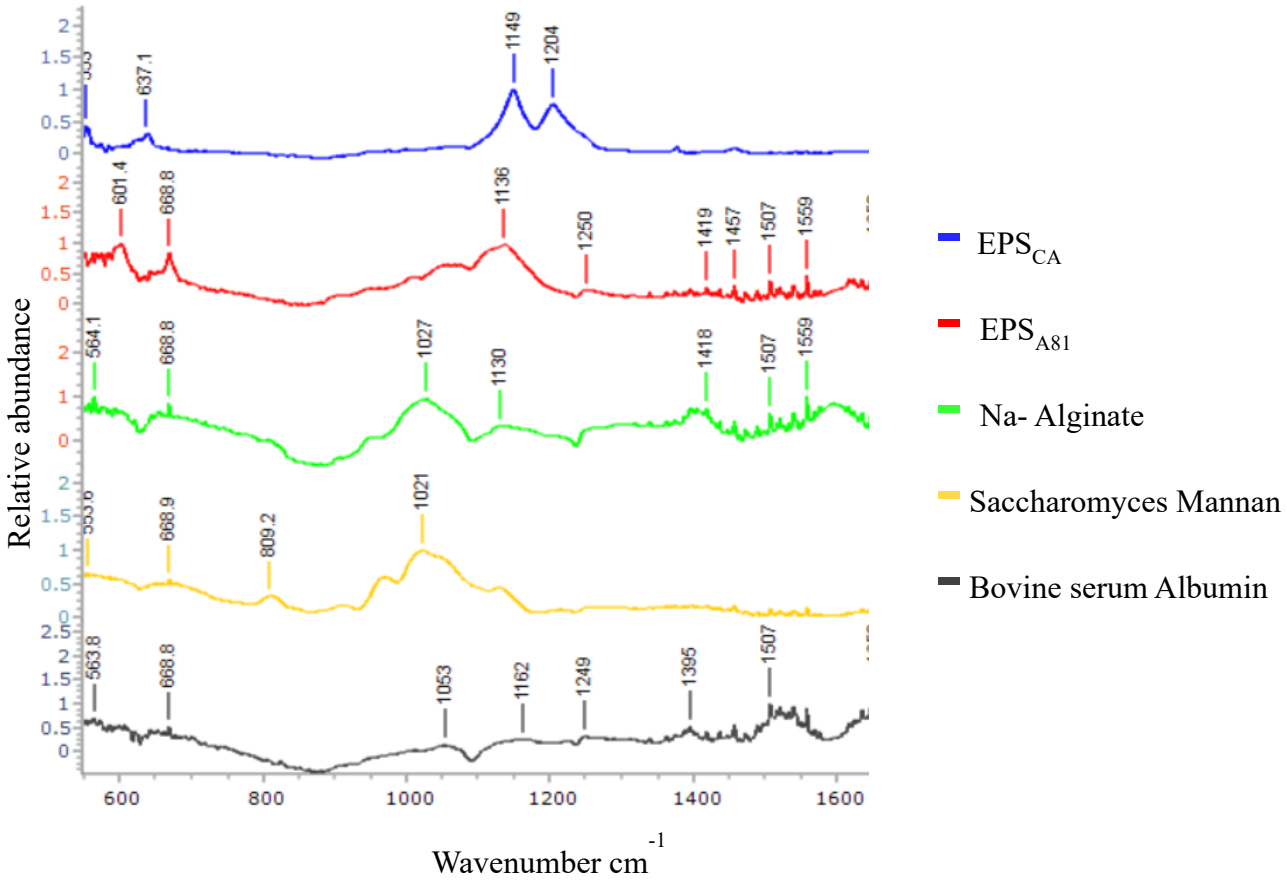


Figure 20. 550-1650cm^{-1} region of the FTIR-ATR spectra. Colors blue, red, green, yellow and black correspond to EPS_{CA}, EPS_{A81}, Na-alginate, Saccharomyces mannan, Bovine serum albumin samples respectively. Peaks finding for X axis was performed at 20% threshold and prominence of 4.

Table 6 (a) Sugar analysis of EPS_{CA}

サンプル名 ： A13705-CA					
記号	成分名	保持時間（分）	pmol	サンプル 1 mg あたり	
				nmol	μg
1	Glucuronic acid	6.6	< 5.0	< 13.3	< 2.6
2	Galacturonic acid	7.1	< 20.0	< 53.1	< 10.3
3	Galactose	10.1	2.9	7.8	1.4
4	Mannose	12.9	12.3	32.8	5.9
5	Glucose	15.0	37.8	100.3	18.1
6	Arabinose	16.1	1.5	4.0	0.6
7	Ribose	18.5	2.9	7.6	1.1
8	N-acetyl-mannosamine	ND	ND	ND	ND
9	Xylose	21.3	7.6	20.1	3.0
10	N-acetyl-glucosamine	22.8	< 1.0	< 2.7	< 0.6
11	Fucose	24.7	< 1.0	< 2.7	< 0.4
12	Rhamnose	28.2	3.6	9.5	1.6
13	N-acetyl-galactosamine	37.6	< 1.0	< 2.7	< 0.6

Table 6 (b) Sugar analysis of EPS_{A81}

サンプル名 ： A12837-A81					
記号	成分名	保持時間（分）	pmol	サンプル 1 mg あたり	
				nmol	μg
1	Glucuronic acid	ND	ND	ND	ND
2	Galacturonic acid	ND	ND	ND	ND
3	Galactose	9.8	3.8	4.5	0.8
4	Mannose	12.6	7.6	9.0	1.6
5	Glucose	14.7	80.4	95.8	17.3
6	Arabinose	15.6	2.3	2.7	0.4
7	Ribose	18.0	8.8	10.5	1.6
8	N-acetyl-mannosamine	ND	ND	ND	ND
9	Xylose	ND	ND	ND	ND
10	N-acetyl-glucosamine	22.4	< 1.0	< 1.2	< 0.3
11	Fucose	ND	ND	ND	ND
12	Rhamnose	27.7	26.4	31.4	5.2
13	N-acetyl-galactosamine	37.2	< 1.0	< 1.2	< 0.3

6.4.3.PGP assay comparing crude EPS

Crude EPS_{CA} exhibited 1.46-folds increase in frond number and 1.51-folds increase in dry weight compared to no EPS control (Figure 21). Mannan from *Sachharomyces* denoted as “M” and Na- alginate had no significant PGP activity.

EPS_{A81} also did not show any PGP activity. Further analysis revealed that crude EPS_{CA} is capable of eliciting PGP effect in concentrations ≥10µg/ml while there is no difference in the PGP effect between concentrations between 10-100µg/ml (Figure 22).

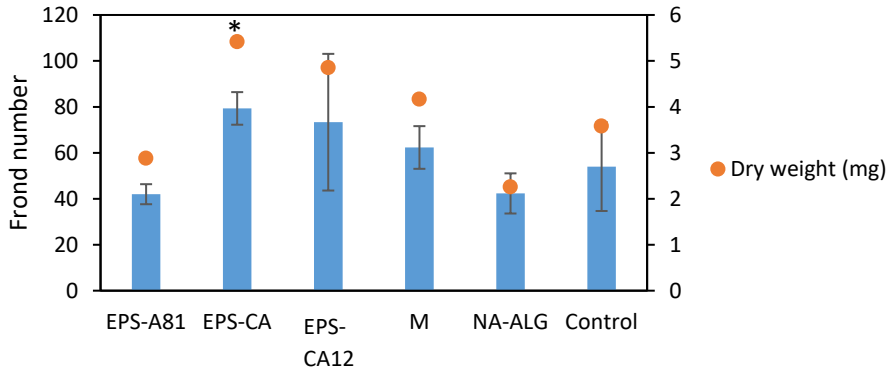


Figure 21. Effect of crude EPS (100µg/ml) on *L. minor* growth. All values are mean ± SD (n = 3). Asterisks indicate the significant differences between control (Student's t-test, * P < 0.05, ** P<0.005). EPS-CA12 is EPS derived from *A. vinelandii* CA12, M is Mannan, NA-ALG is Alginic acid sodium salt and control is non supplemented mH.

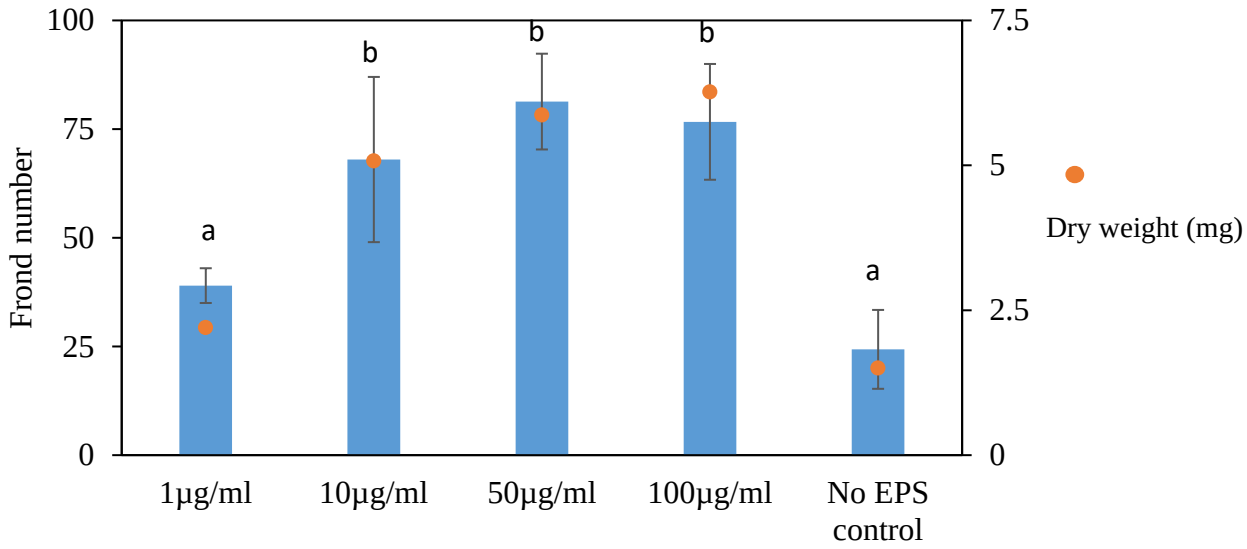


Figure 22. Effect of different concentrations of crude EPS_{CA} on *L. minor*. All values are mean ± SD (n = 3). Different alphabets indicate significant differences (Student's t-test, P < 0.05) between treatments.

6.4.4. PGP assay confirming purified EPS_{CA}

Purified EPS_{CA} retained statistically significant PGP effect at both 10 and 50 µg/ml concentrations larger fronds comparing to no EPS control (Figure 23. a), frond numbers (Figure 23. b) dry weight (Figure 23. c). Further analysis of the spent medium pH revealed that the spent medium acidity had increased significantly (≤ pH 5.0 at day 10 from pH 7 at day 0) correlating to the longer roots compared to control (Figure 23. d).

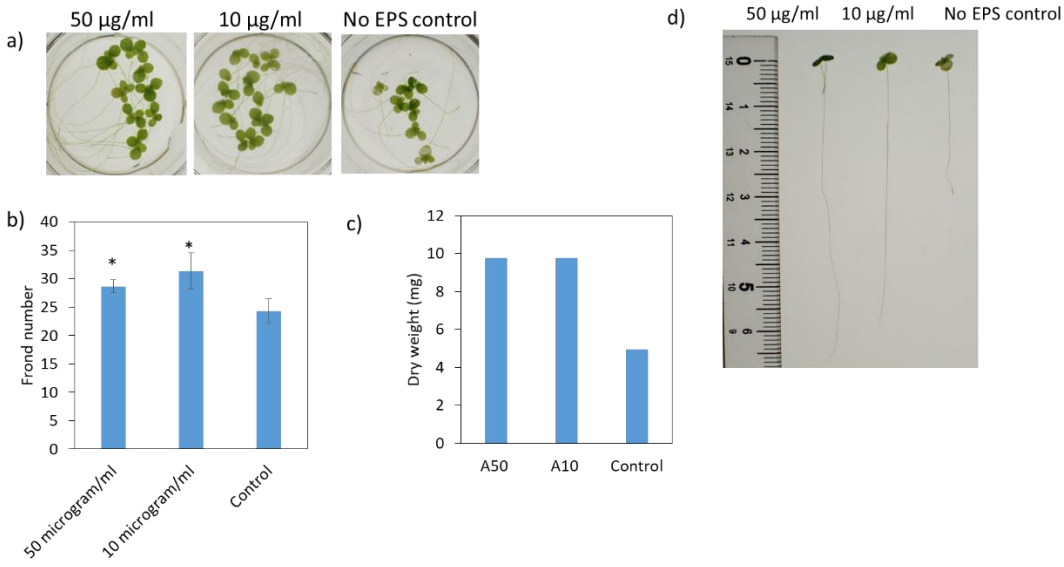


Figure 23. Effect of pure EPS_{CA} on *L. minor* fronds. a) Frond size, b) frond number, c) dry weight, d) root length. A10 and A50 indicate 10 and 50 µg/ml.

6.4.5. PGF assay of alginate lyase lysate of EPS_{A81}

No significant PGP activity of the alginate lyase treated altEPS_{A81} was observed in the frond number but there was 1.12-folds increase in the dry weight compared to control. Alginate lyase enzyme both with Alginic acid- sodium salt substrate and without had a negative effect on the dry weight (Figure 24).

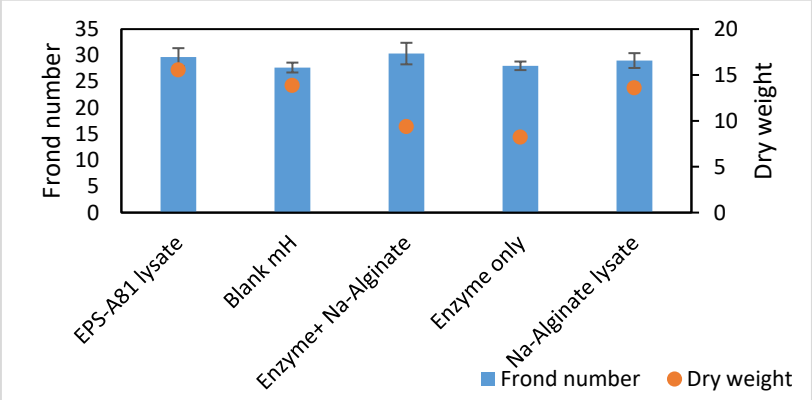


Figure 24. Effect of altEPS$_{A81}$ lysate on *L. minor* growth

6.4.6. Genomic data comparison between A81 and CA

6.4.6.1. Alginate biosynthesis genes

A total of 21 biosynthetic genes were searched for in the whole genome of A81 and CA. Among them, only 10 alginate biosynthesis genes (*alg*A, *alg*D, *alg*44, *alg*V, *alg*F, *alg*G, algE7, *aly*A2, *alg*G) were identical between A81 and CA. Eight genes (*alg*C, *alg*8, *alg*K, *alg*X, *alg*L, *aly*A3, *alg*B, *alg*K) were non-identical between the two genome sequences. Three genes (*alg*U, *alg*T, *alg*E) were not found in either of the genomes. This indicated that the alginate biosynthesis machineries as well as EPS production by A81 and CA may be different.

Table 7: Identification of alginate biosynthesis genes between A81 and CA genome. [✓] indicates the same gene and proteins that were found in the NCBI database. [X] Indicates the genes in the A81 genome that did not have an identical protein in the CA genome. [ND] indicates the genes that were not found in the genome of either strain.

	Gene	*A. vinelandii* CA	*A. vinelandii* A81
1	*alg*A	✓	✓
2	*alg*C	✓	X
3	*alg*D	✓	✓
4	*alg*8	✓	X
5	*alg*44	✓	✓

6	*alg*k	✓	X
7	*alg*U	ND	ND
8	*alg*T	ND	ND
9	*alg*I	✓	✓
10	*alg*V	✓	✓
11	*alg*F	✓	✓
12	*alg*X	✓	X
13	*alg*G	✓	✓
14	*alg*L	✓	X
15	*alg*E7	✓	✓
16	*aly*A2	✓	✓
17	*aly*A3	✓	X
18	*alg*B	✓	X
19	*alg*E	ND	ND
20	*alg*G	✓	✓
21	*alg*K	✓	X

6.4.6.2. Genomic data comparison using JGI IMG/M

Summarized insights from the genomic data comparison using JGI IMG/M is stated bellow:

i. **ANI:** Average Nucleotide Identity (ANI) between CA and A81: 99.66.

ii. Phylogenetic Profilers:

a. **Homologous genes:** Total 4,407 homologous genes found in between CA and A81 genomes. From the homologous genes between CA and A81, thiol: disulfide interchange protein DsbB has the least percent of identity (42.9%). Some of the genes involved in transport of molecules across cell such as outer membrane porin OprD family, Outer membrane transport energization protein TonB, genes involved in producing mannose-6-phosphate isomerase- cupin superfamily have <95% identity between the two genomes. A list of these genes is added to appendix (Table S1)

b. **Genes without homologues:** Total 706 unique genes were found in CA which did not have any homologues in A81 genome. Few of the unique genes found in the CA were Poly (beta-D-mannuronate) C5 epimerase 3,

type IV secretion system protein (VirB2, VirB3, VirB4, VirB5, VirB11, VirB9, VirD2, and VirD4). A complete list is added to appendix (Table S2)

iii. **Abundance profile of overall genes:** Genes uniquely found in CA that are involved in Sulfur and carbohydrate metabolism was analyzed with reference to KEGG database (Table 8).

Table 8: Abundance profile (gene count) between CA and A81 genomes.

Abundance profile overview (Gene count)			
Function Id	**Function Name**	**CA**	**A81**
	Carbohydrate metabolism		
EC:5.4.2.12	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)	2	0
EC:2.7.1.17	Xylulokinase	1	0
EC:3.1.3.8	3-phytase	1	0
	Sulfur metabolism		
EC:4.4.1.13	Cysteine-S-conjugate beta-lyase	1	0
EC:1.1.1.313	Sulfoacetaldehyde reductase	2	0
EC:2.5.1.48	Cystathionine gamma-synthase	1	0

iv. **Abundance of glucosyltransferase genes:** CA genome was found bearing a single additional glucosyltransferase gene which was not present in A81. The protein from this gene is called AvCA_15980 (Figure S12) and blast results show conserved domains in *A. vinelandii* (Figure S13, S14). Further analysis is required to confirm if these differences can explain the EPS structure and chemical composition of CA and A81.

6.5. Discussion

6.5.1. Effect of EPS on the growth of *L. minor*

Metabolically active bacterial colonies on *L. minor* may act as a stationary sources of growth promoters. In a mutually beneficial stable symbiosis, duckweed may in several ways provide nutrition to bacteria to continuously prompt the production of such compounds. We tried to identify if addition of bacterial extracellular polymeric substances may have any growth promotion effect on *L. minor*. PGP activity of widely potential plant growth promoting compounds such as

Saccharomyces yeast mannan and sodium alginate were tested. Bacterial EPS with the antioxidant properties (Wang et al., 2021) has the ability to provide immunity against plant pathogens (Leroux et al., 2011). Moreover, mannan has been reported to interact with plants where plants also modify the intermediate compounds produced by the yeast (Voiniciuc et al., 2019). Na- alginate provides growth promotion by encapsulation of microbes inside its cross linked polymer (Strobel et al., 2018; Vassilev et al., 2020), alleviating oxidative stress during draught condition (Sá et al., 2019). In this experiment, we found no significant PGP activity from EPS_{A81}, mannan and Na- alginate these polymers. Rather, the pure EPS_{CA} showed a significantly high PGP effect on *L. minor* at a concentration as low as ≥10 µg/ml.

6.5.2. Strain specific variations in EPS compositions attributes to the PGP effect on duckweed

Analysis of the EPS_{A81} and EPS_{CA} revealed that these the EPS sugar composition may be different. Significant biofilm production (1.4 OD_{595}) and alginate production (187.6 µg) per OD_{600} at 96 hours indicate A81 strain is metabolically different from CA strain which produced 0.41 OD_{595} biofilm and 1.8 µg per OD_{600} at 96 hours. The same pattern was reflected in the EPS produced by the strains as the alginate concentration of crude EPS_{A81} 79.5% where it was 3.1% for the EPS_{CA} of the dry weight. Genome analysis also help explain the phenotypic difference. It was found that 44% of the alginate biosynthetic genes between A81 and CA genome produce non-identical proteins (Table 7). Further strengthening the fact, the chemical composition and biological activity of the EPS of these strains are possibly different. An analysis on the monomeric sugar composition is also ongoing, which may help elucidate underlying molecular differences elicit PGP effect on *L. minor*. Sugar analysis (Table 6) and FTIR analysis (Figure 20) both suggests the major differences between the EPS of these two strains. Genomic analysis also identifies some tentative candidate genes for future PGP factor analysis (Table S1, S2).

6.5.3. altEPS$_{A81}$ had no PGP effect on *L. minor*

Lyase lysate increased the root length of banana roots (Cao et al., 2007), barley under hypoxic conditions (Tomoda et al., 1994). There is a significant number of studies that show plant growth promotion effect from alginate oligo saccharides (C. Zhang et al., 2020). Alginate oligosaccharides have been reported to have PGP effect on numerous terrestrial plants at 0.02- 1 mg/ml concentrations and can be derived by γ-irradiation (Hien et al., 2000) degradation by bacterial lyase (Iwasaki & Matsubara, 2000), acid hydrolysis of alginate polymers . Alginate is made up of Poly G Poly (D-glucuronic acid) blocks (PG), poly (D-mannuronic acid) blocks (PM), and alternating blocks of D-glucuronic and D-mannuronic residues (GM). Plant growth promotion was only observed when PG and PM was mixed at a certain ratio (X. Xu et al., 2003). This indicates that the PG and PM ratio of alginate produced by different bacterial strains are possibly different which needs further analysis to conclude on the duckweed growth promotion mechanism by the different bacterial EPS. In this experiment, we have utilized enzymatic degradation of EPS$_{A81}$ and found that the bacteria originated EPS has a better growth promotion activity in terms of dry weight increase of *L. minor*. The sugar composition, PG, PM ratio of the alginate EPS needs to be elucidated in order to conclude on the PGP effect.

6.6. Conclusion

Different PGP mechanisms of *A. vinelandii* elicited on *L. minor* was studied. It was confirmed that nitrogen fixation is one of the most important PGP factors in nitrogen free medium but in nitrogen containing medium PGP mechanisms other than nitrogen fixation come into play. One such mechanism is bacterial EPS. The effect of the EPS$_{CA}$ was much more clearly visible owing to the EPS$_{A81}$ not showing a PGP effect on *L. minor*. Further treatment of the A81 EPS also failed to activate the PGP effect, indicating that the EPS$_{CA}$ may be a potential PGP factor from CA strain and solidifying the fact that *A. vinelandii* may impart species specific PGP effect on *L. minor*. It is therefore an interesting phenomenon which requires further work in order to pinpoint the basis of the species-specific PGP effect of a soil bacteria on an unrelated plant *L. minor*.

7. General conclusion

This research attempted to establish a successful mutualism between duckweed and non-indigenous nitrogen fixing bacteria and contributed to exploring the practicalities of an artificially attained symbiotic system for future biotechnological applications.

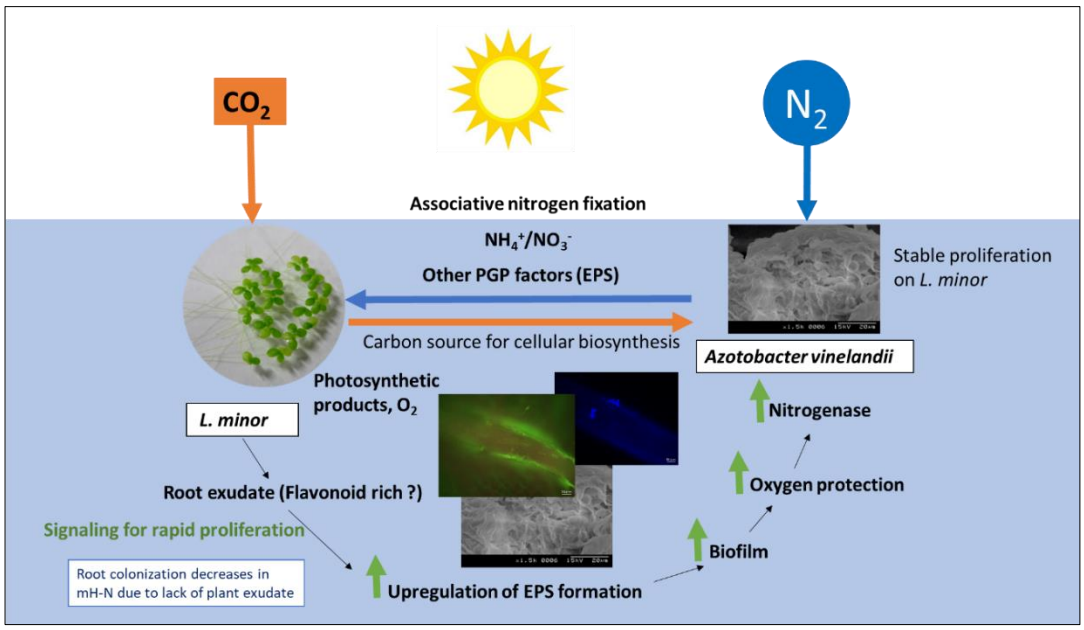


Figure 25: A potential mechanism of mutualistic growth of *A. vinelandii* and *L. minor*

It was found that stable symbiosis enables long term survival of *Azotobacter vinelandii* A81 on *L. minor* even in extreme nutrient scarcity. Nitrogen fixation played a key role in bacterial survival as well as plant growth promotion. The potential application of *A. vinelandii* in providing alternative nitrogen source and cationic stress relief to *L. minor* in low nitrogen wastewater was elucidated. Finally, significant increase in the nitrogenase activity of bacterial culture prompted by the presence of *L. minor* consolidated a mutualistic mechanism between these two organisms.

While the aquatic plant may provide the diazotrophic bacteria an attachment surface, carbon source for cellular biosynthesis and signaling for nitrogen fixation

and rapid proliferation, specific bacterial factors such as EPS provided growth promotion activity to *L. minor* when added extraneously. It was observed that the EPS produced by different strains of *A. vinelandii* are functionally, structurally and chemically different that can be traced back to their genomic data. Further studies into the duckweed- bacteria symbiosis and biologically active bacterial EPS may lead to a deeper understanding of duckweed-microbe interaction in the nature and a more efficient management of duckweed in mass cultivation scenarios.

Appendix

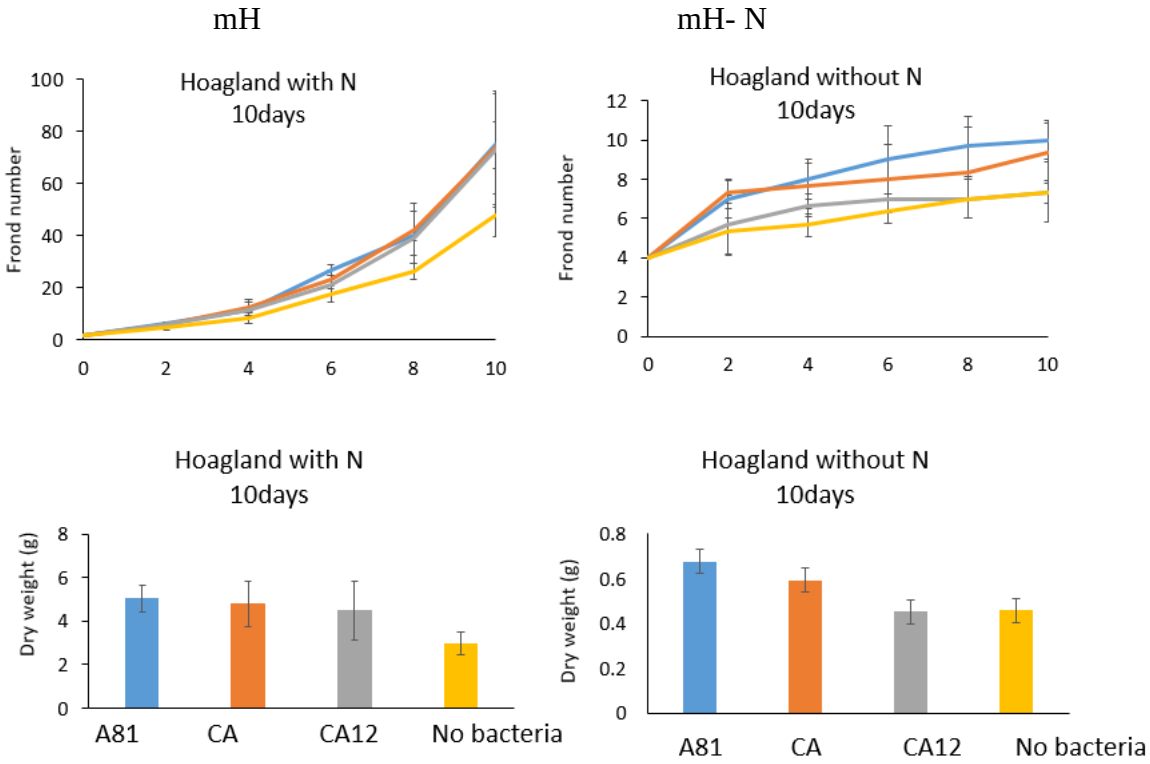


Figure S1: Effect of A. vinelandii strains on the growth of L. minor in mH medium (With and without Nitrogen). Method is described in 2.2.2.1. Colors indicate A81, blue; CA, Orange; CA12, Grey and No bacteria control, yellow. All the duckweed culture experiments were started from two fronds, plants. Values are mean ± SD (n = 3).

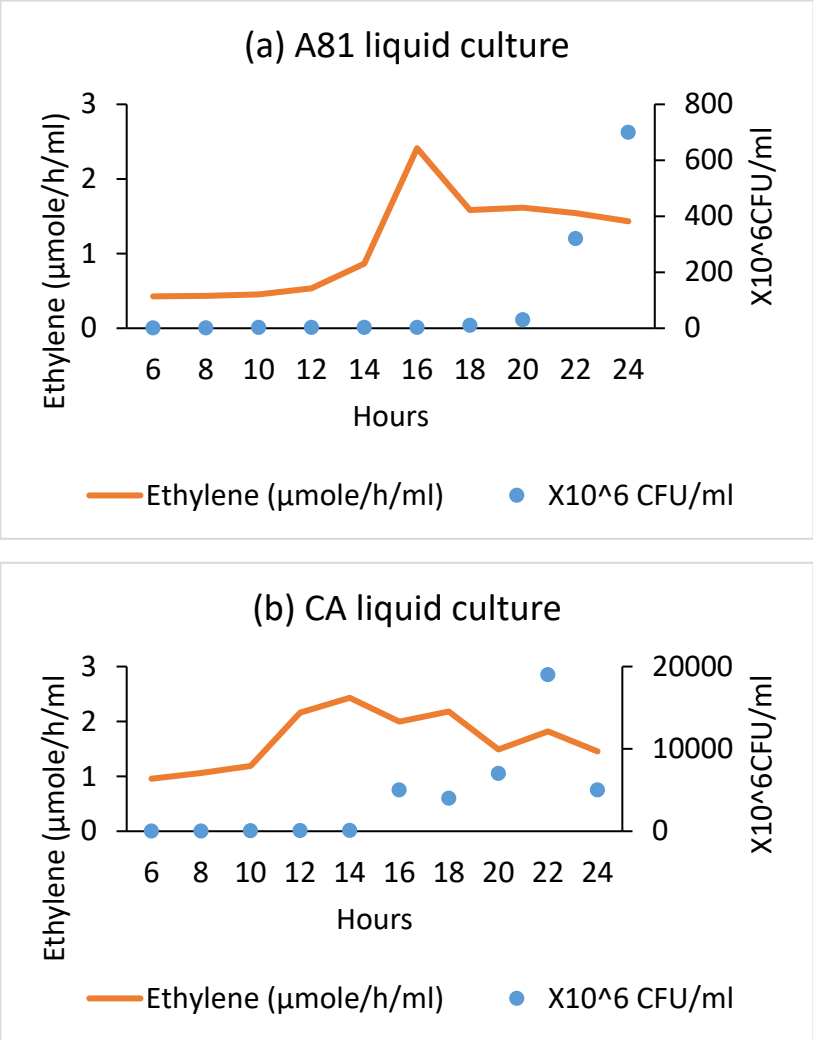


Figure S2: ARA (Acetylene reduction assay) of *A. vinelandii* A81 liquid culture (a), *A. vinelandii* CA liquid culture (b). Samples of 2 ml culture were withdrawn from the culture vessel at 2-hour intervals for 48 hours and taken in a 5ml glass vial. After making the vial airtight, 10% headspace gas was removed and replaced with acetylene (C_2H_2) gas. The vial was immediately incubated at 30°C for 1 hour before taking 200µL gas for analysis in the GC-FID (Shimadzu 2014). Nitrogen fixation activity was quantified using a standard curve made with increasing volume of 803 ppm ethylene gas against the area of the GC peak data.

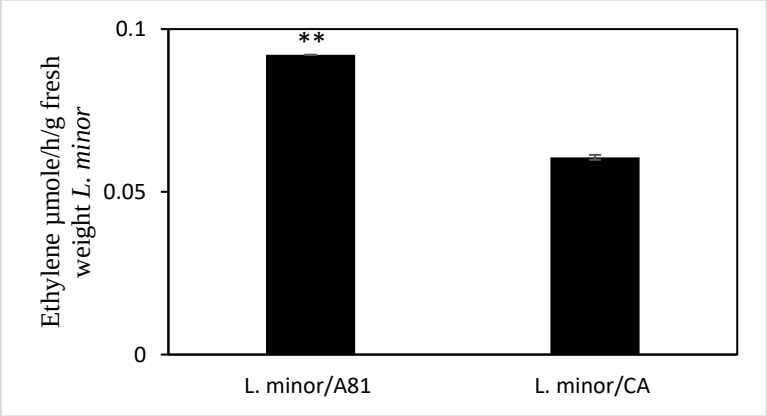


Figure S3. ARA of *A. vinelandii* strains colonized on *L. minor*. Method is described in 2.2.3.5.1. Black bars represent ethylene µmole/h/g fresh weight *L. minor*. All values are mean ± SD (n = 3). Asterisks indicate the significant differences from control (Student's t-test, * P < 0.05, ** P<0.005).

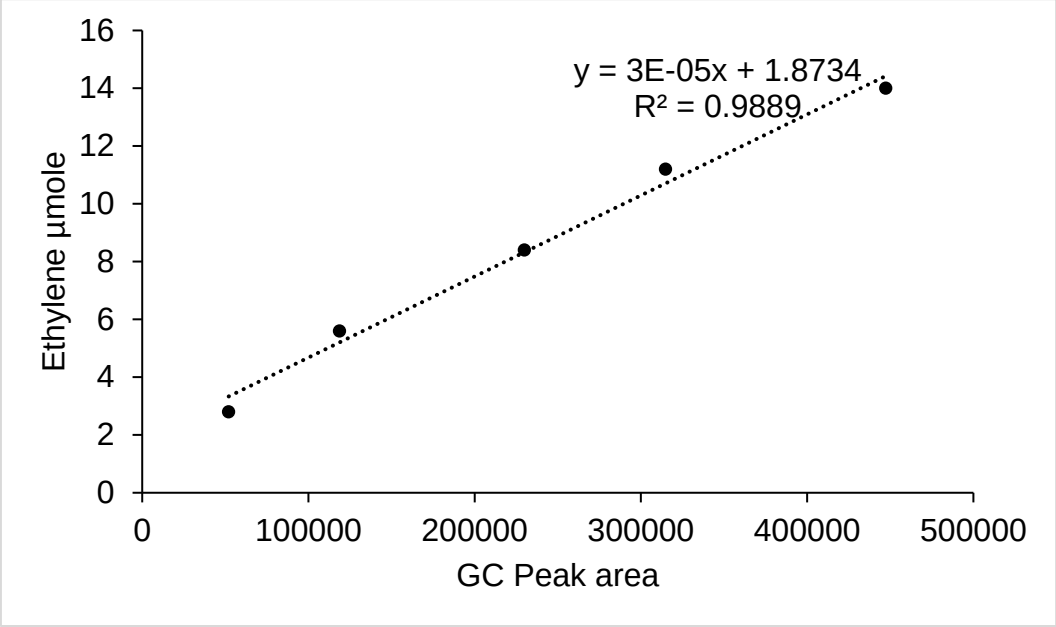


Figure S4: Ethylene standard curve. Constructed by plotting the increasing volume of 803 ppm ethylene (C_2H_4) gas against the area of the GC peak data.

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2022年9月13日

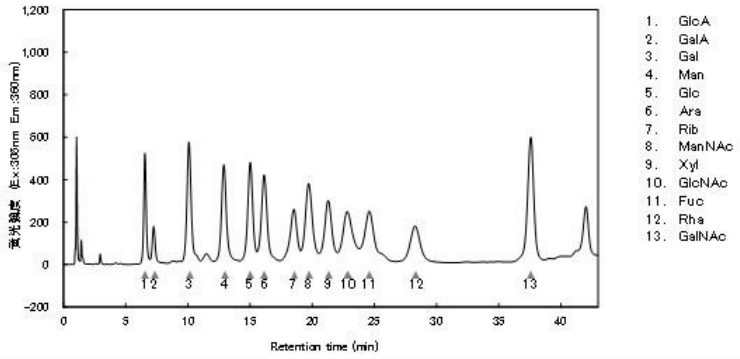
承認	確認	担当

単糖組成分析　レポート

標準単糖(13種類)の分析結果　(N-アセチル化あり)

記号	成分名	分子量	保持時間(分)	分析量
1	Glucuronic acid	194.14	6.5	147.2 pmol
2	Galacturonic acid	194.14	7.3	147.2 pmol
3	Galactose	180.16	10.1	29.4 pmol
4	Mannose	180.16	12.9	29.4 pmol
5	Glucose	180.16	15.0	29.4 pmol
6	Arabinose	150.13	16.1	29.4 pmol
7	Ribose	150.13	18.5	29.4 pmol
8	N-acetyl-mannosamine	221.21	19.7	29.4 pmol
9	Xylose	150.13	21.3	29.4 pmol
10	N-acetyl-glucosamine	221.21	22.8	29.4 pmol
11	Fucose	164.16	24.6	29.4 pmol
12	Rhamnose	164.16	28.3	29.4 pmol
13	N-acetyl-galactosamine	221.21	37.6	29.4 pmol

結果 ID : 2020



前処理工程

酸加水分解:　標準単糖ミクスチャー 50μL に 50μLの 8 M Trifluoroacetic acid を添加し、100℃ で 3 時間の処理をした。

プレラベリング:　無水酢酸を用いて加水分解物の N-アセチル化を行った後に、ABEE化試薬を用いて蛍光標識化を行った。その後、水／クロロホルム抽出により水層から蛍光標識化単糖を回収し、分析に供した。

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Figure S5: EPSCA sugar analysis report standard peaks

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2022年9月13日

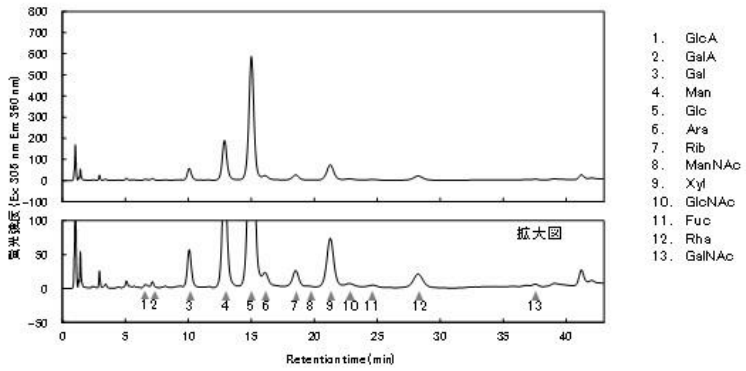
承認	確認	担当

単糖組成分析 レポート

分析日:2022年9月7日	分析番号:ABEE-648	結果 ID: 2022
前処理量:サンプル 50.5μg相当を プレラベリングした。 抽出液量:270μL/tube	分析量: 376.6 ng/50.5 μg 注入量: 20μL	N-アセチル化 : あり

サンプル名 : A13705

記号	成分名	保持時間(分)	pmol	サンプル 1 mg あたり	
				nmol	μg
1	Glucuronic acid	6.6	< 5.0	< 13.3	< 2.6
2	Galacturonic acid	7.1	< 20.0	< 53.1	< 10.3
3	Galactose	10.1	2.9	7.8	1.4
4	Mannose	12.9	12.3	32.8	5.9
5	Glucose	15.0	37.8	100.3	18.1
6	Arabinose	16.1	1.5	4.0	0.6
7	Ribose	18.5	2.9	7.6	1.1
8	N-acetyl-mannosamine	ND	ND	ND	ND
9	Xylose	21.3	7.6	20.1	3.0
10	N-acetyl-glucosamine	22.8	< 1.0	< 2.7	< 0.6
11	Fucose	24.7	< 1.0	< 2.7	< 0.4
12	Rhamnose	28.2	3.6	9.5	1.6
13	N-acetyl-galactosamine	37.6	< 1.0	< 2.7	< 0.6



＜特記事項＞
ND:not detected
＜値:定量下限値未満

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Figure S6: EPS_{CA} sugar analysis report

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INNOVATION BY PROTEIN ENGINEERING

2022年11月14日

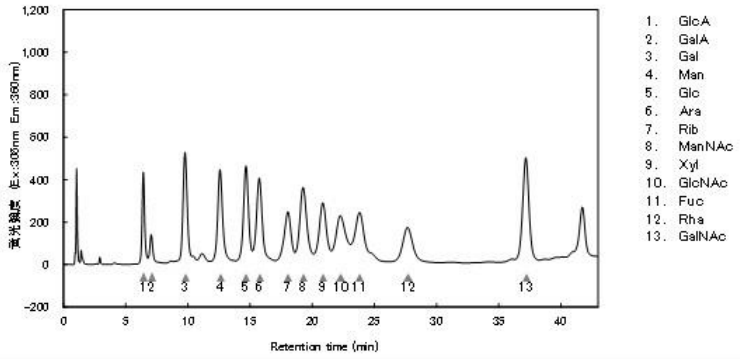
承認	確認	担当

単糖組成分析 レポート

標準単糖(13種類)の分析結果 (N-アセチル化あり)

記号	成分名	分子量	保持時間(分)	分析量
1	Glucuronic acid	194.14	6.4	148.4 pmol
2	Galacturonic acid	194.14	7.1	148.4 pmol
3	Galactose	180.16	9.8	29.7 pmol
4	Mannose	180.16	12.6	29.7 pmol
5	Glucose	180.16	14.7	29.7 pmol
6	Arabinose	150.13	15.7	29.7 pmol
7	Ribose	150.13	18.0	29.7 pmol
8	N-acetyl-mannosamine	221.21	19.3	29.7 pmol
9	Xylose	150.13	20.9	29.7 pmol
10	N-acetyl-glucosamine	221.21	22.3	29.7 pmol
11	Fucose	164.16	23.8	29.7 pmol
12	Rhamnose	164.16	27.7	29.7 pmol
13	N-acetyl-galactosamine	221.21	37.2	29.7 pmol

結果 ID : 2126



前処理工程

酸加水分解: 標準単糖ミクスチャー 50μLに 50μLの 8 M Trifluoroacetic acid を添加し、100℃ で 3 時間の処理をした。

プレラベリング: 無水酢酸を用いて加水分解物の N-アセチル化を行った後に、ABEE化試薬を用いて蛍光標識化を行った。その後、水／クロロホルム抽出により水層から蛍光標識化単糖を回収し、分析に供した。

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Figure S7: EPSA81 sugar analysis report standard peaks

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INNOVATION BY PROTEIN ENGINEERING

2022年11月14日

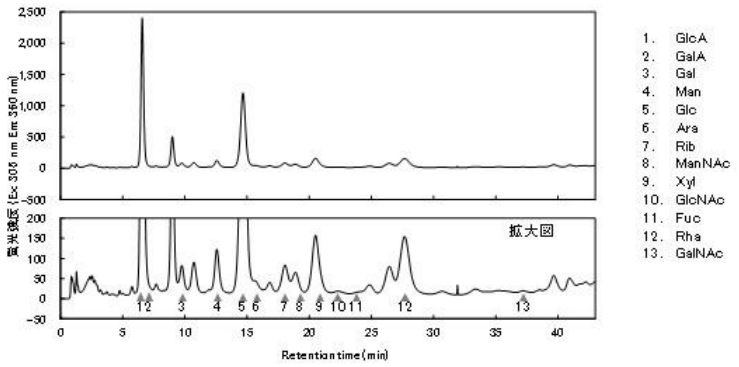
承認	確認	担当

単糖組成分析　レポート

分析日:2022年11月10日	分析番号:ABEE-649	結果 ID: 2128
前処理量:サンプル 565.0μg相当を プレラベリングした。 抽出液量:270μL/tube	分析量: 839.4 ng/565.0 μg 注入量: 20μL	N-アセチル化　: あり

サンプル名　:　A12837

記号	成分名	保持時間(分)	pmol	サンプル 1 mg あたり	
				nmol	μg
1	Glucuronic acid	ND	ND	ND	ND
2	Galacturonic acid	ND	ND	ND	ND
3	Galactose	9.8	3.8	4.5	0.8
4	Mannose	12.6	7.6	9.0	1.6
5	Glucose	14.7	80.4	95.8	17.3
6	Arabinose	15.6	2.3	2.7	0.4
7	Ribose	18.0	8.8	10.5	1.6
8	N-acetyl-mannosamine	ND	ND	ND	ND
9	Xylose	ND	ND	ND	ND
10	N-acetyl-glucosamine	22.4	< 1.0	< 1.2	< 0.3
11	Fucose	ND	ND	ND	ND
12	Rhamnose	27.7	26.4	31.4	5.2
13	N-acetyl-galactosamine	37.2	< 1.0	< 1.2	< 0.3



＜特記事項＞
ND:not detected
＜値:定量下限値未満
保持時間 6.6 分にピークが検出されたが、GlcA　の保持時間(6.4 分)と一致しない。

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Figure S8: EPSA81 sugar analysis report

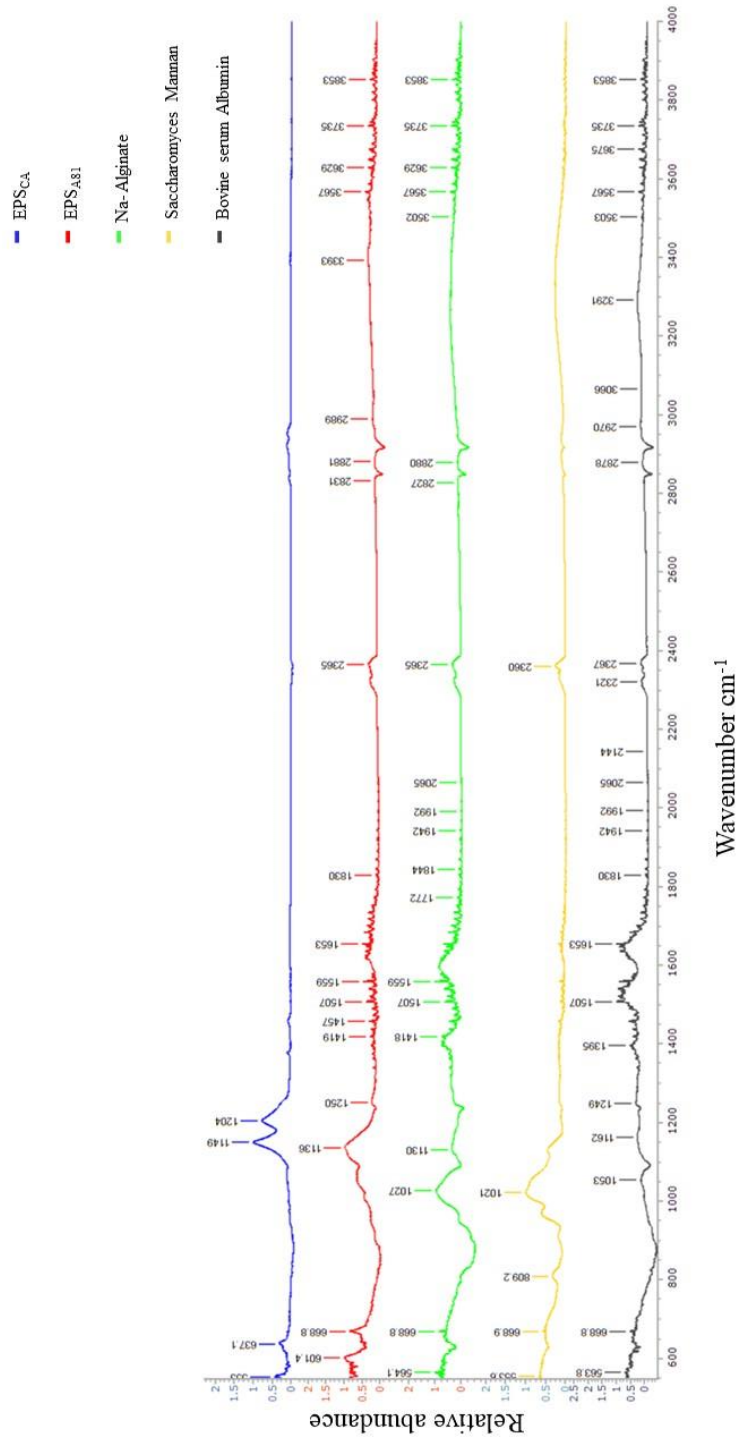


Figure S9: FTIR-ATR spectra (550- 4000 cm^{-1}) of samples used in this study

Select	Domain ▲	Sequencing Status	Study Name	Genome Name / Sample Name	Sequencing Center	IMG Genome ID	Genome Size * assembled	Gene Count * assembled
☐	Bacteria	Finished	Azotobacter vinelandii CA Genome sequencing	Azotobacter vinelandii CA	North Carolina State University	2541047084	5366370	5147
☐	Bacteria	Permanent Draft	Azotobacter vinelandii NBRC 13581 genome sequencing project	Azotobacter vinelandii NBRC 13581	National Institute of Technology and Evaluation	2731957617	5130231	4872

Figure S10: Genomes used for analysis in JGI IMG/M website

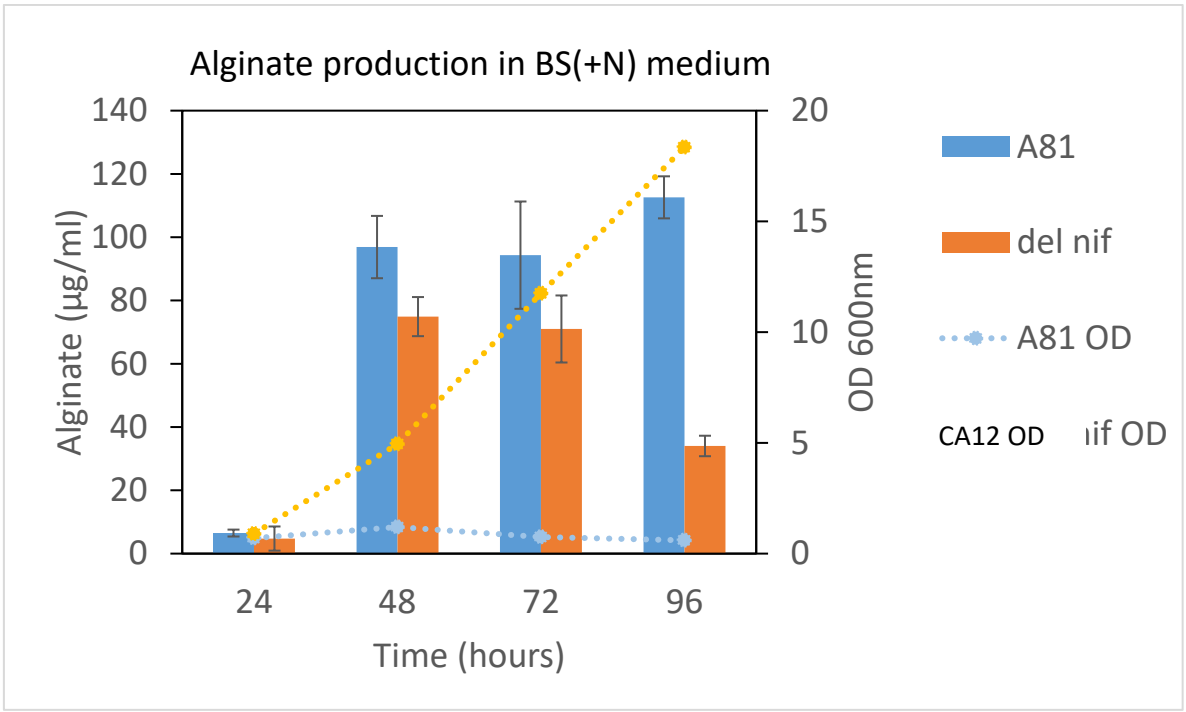


Figure S11: Alginate production profiling of A81 and CA12 over 96hrs. Blue and orange bars indicate Alginate (µg/ml) of A81 and CA12 respectively. Blue and orange lines indicate OD_{600} of A81 and CA12 respectively.

Gene ID	Locus Tag	Product Name	Scaffold ID	Genome
2541769798	AvCA_15980	hypothetical protein	CP005094	Azotobacter vinelandii CA
2541769994	AvCA_17910	alpha-1,6-mannosyltransferase	CP005094	Azotobacter vinelandii CA
2541771192	AvCA_30000	Glycosyltransferase involved in cell wall bisynthesis	CP005094	Azotobacter vinelandii CA
2541772674	AvCA_44840	UDP-glucose:(heptosyl)LPS alpha-1,3-glucosyltransferase	CP005094	Azotobacter vinelandii CA
2541772737	AvCA_45500	membrane glycosyltransferase	CP005094	Azotobacter vinelandii CA
2541772739	AvCA_45520	membrane glycosyltransferase	CP005094	Azotobacter vinelandii CA
2732430793	Ga0128572_10227	UDP-glucose:(heptosyl)LPS alpha-1,3-glucosyltransferase	Ga0128572_1022	Azotobacter vinelandii NBRC 13581
2732431226	Ga0128572_103020	alpha-1,6-mannosyltransferase	Ga0128572_1030	Azotobacter vinelandii NBRC 13581
2732431646	Ga0128572_104119	membrane glycosyltransferase	Ga0128572_1041	Azotobacter vinelandii NBRC 13581
2732431648	Ga0128572_104121	membrane glycosyltransferase	Ga0128572_1041	Azotobacter vinelandii NBRC 13581
2732433557	Ga0128572_11547	Glycosyltransferase Family 4	Ga0128572_1154	Azotobacter vinelandii NBRC 13581

Figure S12: Comparison of the glucosyltransferase proteins found in CA and A81 genomes. Blue highlights AvCA_15980 gene, an additional protein found in CA.

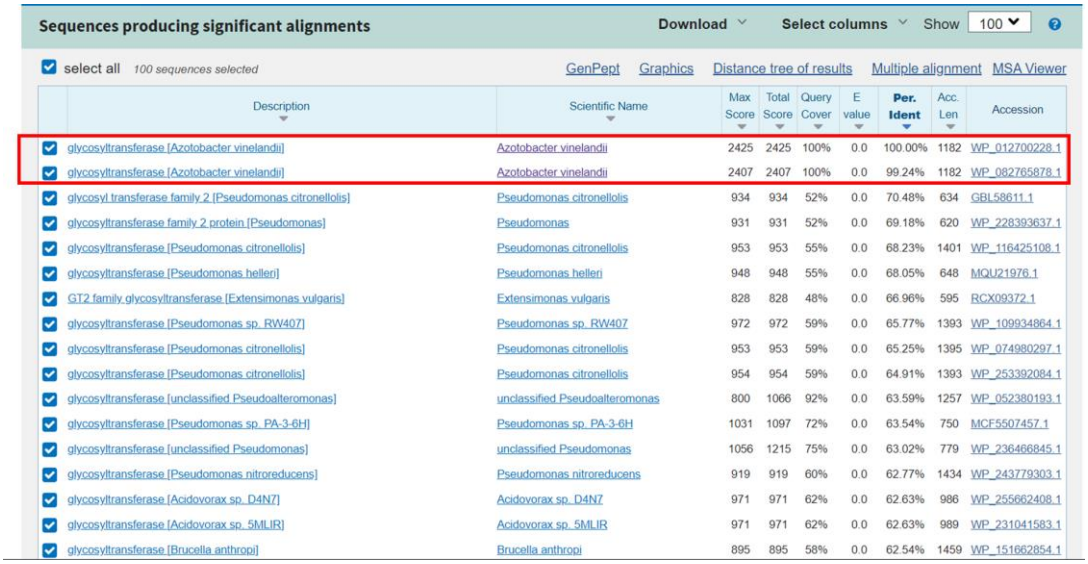
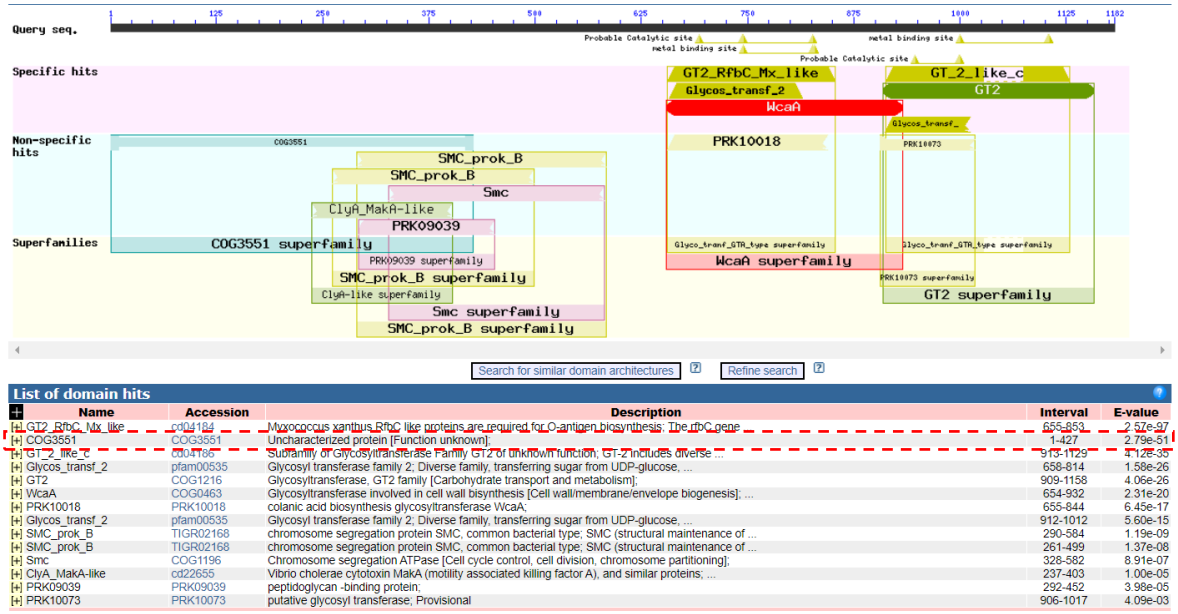


Figure S13: BLAST result of the AvCA_15980 protein identifies it as a glucosyltransferase conserved in *A. vinelandii* genome with nearest identical gene (70.48%) in *Pseudomonas citronellois*.



List of domain hits

Name	Accession	Description	Interval	E-value
[+] GT2_RfbC_Mx_like	cd04184	Myxococcus xanthus RfbC like proteins are required for O-antigen biosynthesis; The rfbC gene	656-853	2.57e-97
[+] COG3551	COG3551	Uncharacterized protein [Function unknown];	1-427	2.79e-51
[+] GT_2_like_c	cd04186	Subfamily of Glycosyltransferase Family GT2 of unknown function; GT-2 includes diverse ...	913-1129	4.12e-35
[+] Glycos_transf_2	pfam00535	Glycosyl transferase family 2; Diverse family, transferring sugar from UDP-glucose, ...	658-814	1.58e-26
[+] GT2	COG1216	Glycosyltransferase, GT2 family [Carbohydrate transport and metabolism];	909-1158	4.06e-26
[+] WcaA	COG0463	Glycosyltransferase involved in cell wall biosynthesis [Cell wall/membrane/envelope biogenesis]; ...	654-932	2.31e-20
[+] PRK10018	PRK10018	colanic acid biosynthesis glycosyltransferase WcaA;	655-844	6.45e-17
[+] Glycos_transf_2	pfam00535	Glycosyl transferase family 2; Diverse family, transferring sugar from UDP-glucose, ...	912-1012	5.60e-15
[+] SMC_prok_B	TIGR02168	chromosome segregation protein SMC, common bacterial type; SMC (structural maintenance of ...	290-584	1.19e-09
[+] SMC_prok_B	TIGR02168	chromosome segregation protein SMC, common bacterial type; SMC (structural maintenance of ...	261-499	1.37e-08
[+] Smc	COG1196	Chromosome segregation ATPase [Cell cycle control, cell division, chromosome partitioning];	328-582	8.91e-07
[+] ClyA_MakA-like	cd22655	Vibrio cholerae cytotoxin MakA (motility associated killing factor A), and similar proteins; ...	237-403	1.00e-05
[+] PRK09039	PRK09039	peptidoglycan -binding protein;	292-452	3.98e-05
[+] PRK10073	PRK10073	putative glycosyl transferase; Provisional	906-1017	4.09e-03

Figure S14: Conserved regions of AvCA_15980 with predicted protein motifs. Nucleotide sequence 1-427 is highlighted by dotted red box which shows an uncharacterized protein.

Table S1: CA genome-Homologous found in A81. Only genes with <95% identity is shown

Result	Gene ID	Locus Tag	Gene Name	Length	Percent Identity
836	2541769169	AvCA_09724	Thiol: disulfide interchange protein DsbB	165	42.9
719	2541769023	AvCA_08180	DNA-binding transcriptional regulator, LysR family	317	46.7
833	2541769158	AvCA_09610	transposase, IS111A/IS1328/IS1533	33	66.7
81	2541768290	AvCA_00920	outer membrane porin, OprD family	423	70.2
949	2541769297	AvCA_11010	Small hydrophilic protein	133	73.7
832	2541769157	AvCA_09600	transposase, IS4 family	245	74.3
320	2541768550	AvCA_03540	ATP-dependent RNA helicase RhlE	557	79.7
433	2541768691	AvCA_04900	hypothetical protein	253	84.4
442	2541768701	AvCA_05000	outer membrane transport energization protein TonB	290	87.6
605	2541768885	AvCA_06830	hypothetical protein	95	88
834	2541769159	AvCA_09620	hypothetical protein	38	88.5
837	2541769171	AvCA_09750	Transposase InsO and inactivated derivatives	286	88.7
831	2541769156	AvCA_09590	protein of unknown function (DUF4198)	311	89.1
890	2541769230	AvCA_10330	phosphoribosyl-dephospho-CoA transferase	212	89.6
835	2541769160	AvCA_09630	hypothetical protein	46	91.3
368	2541768606	AvCA_04110	hypothetical protein	87	92.5
117	2541768328	AvCA_01320	Mannose-6-phosphate isomerase, cupin superfamily	122	94.4
889	2541769229	AvCA_10320	malonate decarboxylase gamma subunit	268	94.4
748	2541769056	AvCA_08510	hypothetical protein	121	95

Table S2: CA genome-Non Homologous found in A81

Result	Gene ID	Locus Tag	Gene Name	Length
688	2541773290	AvCA_51170	Poly(beta-D-mannuronate) C5 epimerase 3	1839
110	2541769164	AvCA_09690	amino acid adenylation domain-containing protein	1388
109	2541769163	AvCA_09680	amino acid adenylation domain-containing protein	1292
694	2541773353	AvCA_51880	AAA domain-containing protein	1212
185	2541769798	AvCA_15980	hypothetical protein	1182
532	2541771934	AvCA_37360	phage tail tape measure protein, TP901 family, core region	1078
664	2541773148	AvCA_49670	Phage integrase family protein	1041
676	2541773161	AvCA_49810	Uncharacterized protein YPO0396	979
703	2541773402	AvCA_52390	Helicase conserved C-terminal domain-containing protein	929
698	2541773396	AvCA_52330	type III restriction enzyme	923
517	2541771917	AvCA_37190	Toprim-like	857
452	2541771766	AvCA_35690	type IV secretion system protein VirB4	816
483	2541771805	AvCA_36080	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase	723
361	2541771301	AvCA_31060	hypothetical protein	705
457	2541771771	AvCA_35740	type IV secretion system protein VirD4	666
700	2541773399	AvCA_52360	hypothetical protein	665
376	2541771358	AvCA_31630	CRISPR-associated helicase, Cas3 family	661
469	2541771790	AvCA_35930	Type IV secretory pathway, VirD2 components (relaxase)	661
589	2541772370	AvCA_41800	FAD-NAD(P)-binding	615
693	2541773352	AvCA_51870	Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthase	613
111	2541769165	AvCA_09700	transposase, IS4 family	610
606	2541772506	AvCA_43140	phage integrase-like protein	602
374	2541771356	AvCA_31610	CRISPR-associated protein, Csd1 family	598
287	2541770670	AvCA_24740	group II intron reverse transcriptase/maturase	591
200	2541769896	AvCA_16940	Protein of unknown function (DUF3631)	571
499	2541771830	AvCA_36320	serine/threonine protein kinase	566
672	2541773156	AvCA_49760	hypothetical protein	563
699	2541773398	AvCA_52350	adenine-specific DNA-methyltransferase	560
113	2541769167	AvCA_09720	ATP-binding cassette, subfamily B	553
107	2541769161	AvCA_09640	ATP-binding cassette, subfamily B	550
512	2541771912	AvCA_37130	Site-specific DNA recombinase	520
671	2541773155	AvCA_49750	Fic/DOC family protein	509
480	2541771802	AvCA_36050	DNA (cytosine-5)-methyltransferase 1	501
459	2541771774	AvCA_35770	MFS transporter, DHA2 family, multidrug resistance protein	492
668	2541773152	AvCA_49720	hypothetical protein	490
147	2541769421	AvCA_12240	Transposase DDE domain-containing protein	460
220	2541770030	AvCA_18270	Transposase DDE domain-containing protein	460
222	2541770036	AvCA_18340	Transposase DDE domain-containing protein	460
363	2541771326	AvCA_31320	Transposase DDE domain-containing protein	460
407	2541771524	AvCA_33340	Transposase DDE domain-containing protein	460
449	2541771763	AvCA_35660	type IV secretion system protein TrbL	457
678	2541773 163	AvCA_49830	Protein of unknown function (DUF3375)	450

482	2541771804	AvCA_36070	hypothetical protein	447
591	2541772372	AvCA_41820	L-lysine 2,3-aminomutase	433
553	2541771960	AvCA_37630	hypothetical protein	426
538	2541771941	AvCA_37430	BNR domain-containing protein	418
588	2541772369	AvCA_41790	Predicted arabinose efflux permease, MFS family	415
695	2541773373	AvCA_52080	hypothetical protein	407
184	2541769778	AvCA_15780	putative transposase	406
9	2541768273	AvCA_00750	putative transposase	403
195	2541769879	AvCA_16800	transposase, IS605 OrfB family, central region	403
603	2541772424	AvCA_42370	transposase, IS605 OrfB family, central region	403
465	2541771783	AvCA_35860	Predicted arabinose efflux permease, MFS family	402
530	2541771932	AvCA_37340	hypothetical protein	402
486	2541771809	AvCA_36120	Integrase	400
466	2541771785	AvCA_35880	CubicO group peptidase, beta-lactamase class C family	396
536	2541771938	AvCA_37400	hypothetical protein	396
467	2541771787	AvCA_35900	Predicted arabinose efflux permease, MFS family	392
509	2541771852	AvCA_36540	Fic family protein	374
169	2541769592	AvCA_14000	hypothetical protein	366
296	2541770745	AvCA_25460	Protein N-acetyltransferase, RimJ/RimL family	352
455	2541771769	AvCA_35720	type IV secretion system protein VirB11	350
371	2541771353	AvCA_31580	CRISPR-associated protein, Cas1 family	346
461	2541771776	AvCA_35790	Major royal jelly protein	343
102	2541769150	AvCA_09530	Transposase	341
368	2541771350	AvCA_31560	Transposase	341
505	2541771846	AvCA_36480	Transposase	341
660	2541773144	AvCA_49630	Transposase	341
251	2541770392	AvCA_21970	NitT/TauT family transport system substrate-binding protein	330
665	2541773149	AvCA_49680	hypothetical protein	329
165	2541769562	AvCA_13670	Transposase	317
250	2541770391	AvCA_21960	taurine dioxygenase	317
701	2541773400	AvCA_52370	protein of unknown function (DUF4868)	317
501	2541771840	AvCA_36420	Transposase	311
592	2541772373	AvCA_41840	DNA-binding transcriptional regulator, LysR family	311
604	2541772450	AvCA_42580	Uncharacterized iron-regulated protein	308
541	2541771944	AvCA_37460	Phage-related baseplate assembly protein	305
373	2541771355	AvCA_31600	CRISPR-associated protein, Csd2 family	302
527	2541771929	AvCA_37310	protein of unknown function (DUF955)	302
539	2541771942	AvCA_37440	Phage tail-collar fibre protein	299
447	2541771761	AvCA_35640	type IV secretion system protein VirB9	298
474	2541771795	AvCA_35980	Plasmid replication initiator protein	295
202	2541769908	AvCA_17060	SIR2-like domain-containing protein	294
462	2541771777	AvCA_35800	Ketosteroid isomerase-related protein	287
654	2541773067	AvCA_48810	hypothetical protein	287
525	2541771927	AvCA_37290	Protein of unknown function (DUF2971)	284
552	2541771957	AvCA_37600	Phage capsid scaffolding protein (GPO) serine peptidase	277
548	2541771951	AvCA_37540	Putative peptidoglycan binding domain-containing protein	276
252	2541770394	AvCA_21990	NitT/TauT family transport system permease protein	273

25	2541768537	AvCA_03410	hypothetical protein	271
704	2541773403	AvCA_52400	restriction system protein	271
540	2541771943	AvCA_37450	phage tail protein, P2 protein I family	266
300	2541770751	AvCA_25530	Integrase core domain-containing protein	265
590	2541772371	AvCA_41810	Glyoxylase, beta-lactamase superfamily II	265
476	2541771797	AvCA_36000	hypothetical protein	260
103	2541769151	AvCA_09540	DNA replication protein DnaC	259
367	2541771349	AvCA_31550	DNA replication protein DnaC	259
504	2541771845	AvCA_36470	DNA replication protein DnaC	259
661	2541773145	AvCA_49640	DNA replication protein DnaC	259
402	2541771480	AvCA_32880	glutamine amidotransferase	257
360	2541771295	AvCA_31000	3-oxoacyl-[acyl-carrier protein] reductase	255
652	2541773063	AvCA_48770	5-methylcytosine-specific restriction enzyme A	247
451	2541771765	AvCA_35680	P-type conjugative transfer protein TrbJ	245
108	2541769162	AvCA_09670	thiol:disulfide interchange protein DsbA	242
632	2541772685	AvCA_44950	hypothetical protein	238
112	2541769166	AvCA_09710	Lanthionine synthetase C-like protein	236
146	2541769420	AvCA_12230	ISXO2-like transposase domain-containing protein	236
503	2541771842	AvCA_36440	hypothetical protein	235
448	2541771762	AvCA_35650	type IV secretion system protein VirB5	234
596	2541772382	AvCA_41930	Transposase DDE domain-containing protein	234
551	2541771955	AvCA_37580	Phage small terminase subunit	229
375	2541771357	AvCA_31620	CRISPR-associated protein, Cas5d family	224
345	2541771121	AvCA_29270	hypothetical protein	220
677	2541773162	AvCA_49820	protein of unknown function (DUF4194)	220
5	2541768268	AvCA_00700	Transposase	213
159	2541769550	AvCA_13540	Transposase	213
160	2541769553	AvCA_13570	Transposase	213
205	2541769918	AvCA_17150	Transposase	213
249	2541770373	AvCA_21760	Transposase	213
276	2541770590	AvCA_23930	Transposase	213
292	2541770711	AvCA_25130	Transposase	213
353	2541771213	AvCA_30220	Transposase	213
414	2541771545	AvCA_33560	Transposase	213
629	2541772657	AvCA_44670	Transposase	213
635	2541772766	AvCA_45800	Transposase	213
638	2541772799	AvCA_65260	Transposase	213
473	2541771794	AvCA_35970	plasmid segregation oscillating ATPase ParF	212
502	2541771841	AvCA_36430	hypothetical protein	212
460	2541771775	AvCA_35780	transcriptional regulator, TetR family	205
114	2541769168	AvCA_09722	thiol:disulfide interchange protein DsbA	201
104	2541769152	AvCA_09550	Transposase	200
372	2541771354	AvCA_31590	CRISPR-associated exonuclease, Cas4 family	199
378	2541771360	AvCA_31650	LysR substrate binding domain-containing protein	190
608	2541772508	AvCA_43160	Site-specific DNA recombinase	189
355	2541771273	AvCA_30776	Uracil DNA glycosylase superfamily protein	188
609	2541772509	AvCA_43170	hypothetical protein	185

463	2541771778	AvCA_35810	transcriptional regulator, TetR family	183
520	2541771921	AvCA_37230	anti-repressor protein	182
543	2541771946	AvCA_37480	phage baseplate assembly protein V	180
569	2541772181	AvCA_39850	hypothetical protein	180
545	2541771948	AvCA_37500	P2 phage tail completion protein R (GpR)	176
470	2541771791	AvCA_35940	conjugative transfer signal peptidase TraF	174
131	2541769325	AvCA_11310	hypothetical protein	173
2	2541768249	AvCA_00510	hypothetical protein	171
535	2541771937	AvCA_37390	hypothetical protein	171
197	2541769893	AvCA_16910	hypothetical protein	169
487	2541771810	AvCA_36130	Transposase	169
143	2541769406	AvCA_12104	type IV pilus assembly protein PilA	165
507	2541771850	AvCA_36520	hypothetical protein	158
157	2541769548	AvCA_13510	Homeodomain-like domain-containing protein	157
162	2541769555	AvCA_13600	Homeodomain-like domain-containing protein	157
189	2541769851	AvCA_16510	Homeodomain-like domain-containing protein	157
235	2541770273	AvCA_20700	Homeodomain-like domain-containing protein	157
283	2541770653	AvCA_24580	Homeodomain-like domain-containing protein	157
298	2541770747	AvCA_25480	Homeodomain-like domain-containing protein	157
356	2541771274	AvCA_30780	Homeodomain-like domain-containing protein	157
583	2541772349	AvCA_41580	Homeodomain-like domain-containing protein	157
667	2541773151	AvCA_49710	Homeodomain-like domain-containing protein	157
310	2541770834	AvCA_26400	hypothetical protein	156
528	2541771930	AvCA_37320	Protein of unknown function (DUF2442)	155
456	2541771770	AvCA_35730	hypothetical protein	154
550	2541771954	AvCA_37570	Phage head completion protein (GPL)	154
471	2541771792	AvCA_35950	Protein of unknown function (DUF2840)	152
600	2541772412	AvCA_42240	hypothetical protein	152
415	2541771546	AvCA_33570	hypothetical protein	151
544	2541771947	AvCA_37490	phage virion morphogenesis (putative tail completion) protein	151
163	2541769559	AvCA_13640	toxin CptA	150
587	2541772368	AvCA_41770	4-carboxymuconolactone decarboxylase	149
531	2541771933	AvCA_37350	hypothetical protein	147
390	2541771426	AvCA_32320	Transposase	146
523	2541771924	AvCA_37260	hypothetical protein	145
636	2541772767	AvCA_45810	Cache domain	145
66	2541768912	AvCA_07100	hypothetical protein	143
206	2541769919	AvCA_17160	putative conserved	141
241	2541770304	AvCA_21020	hypothetical protein	141
295	2541770742	AvCA_25430	hypothetical protein	140
547	2541771950	AvCA_37530	phage lysis regulatory protein, LysB family	140
687	2541773288	AvCA_51140	hypothetical protein	139
642	2541772837	AvCA_46490	hypothetical protein	137
491	2541771816	AvCA_36180	tRNA(fMet)-specific endonuclease VapC	136
105	2541769154	AvCA_09570	Transposase	135
126	2541769279	AvCA_10830	Transposase	135
177	2541769699	AvCA_15020	Putative transposase of IS4/5 family (DUF4096)	135

278	2541770592	AvCA_23950	Transposase	135
405	2541771522	AvCA_33320	Transposase	135
489	2541771814	AvCA_36164	Transposase	135
681	2541773166	AvCA_49860	Transposase	135
255	2541770413	AvCA_22180	hypothetical protein	132
514	2541771914	AvCA_37150	Protein of unknown function (DUF2528)	132
428	2541771592	AvCA_34030	hypothetical protein	130
496	2541771821	AvCA_36230	conjugative transfer region protein, TIGR03750 family	130
134	2541769355	AvCA_11610	hypothetical protein	127
398	2541771454	AvCA_32610	Transposase	127
144	2541769407	AvCA_12107	hypothetical protein	126
88	2541769052	AvCA_08470	hypothetical protein	125
434	2541771649	AvCA_34540	hypothetical protein	125
99	2541769147	AvCA_09490	LysR substrate binding domain-containing protein	121
155	2541769530	AvCA_13330	hypothetical protein	120
477	2541771798	AvCA_36010	Protein of unknown function (DUF2958)	118
493	2541771818	AvCA_36200	integrative conjugative element protein, RAQPRD family	118
518	2541771919	AvCA_37210	hypothetical protein	117
542	2541771945	AvCA_37470	hypothetical protein	117
43	2541768656	AvCA_04560	hypothetical protein	116
331	2541771005	AvCA_28090	Transposase	116
610	2541772510	AvCA_43180	hypothetical protein	116
187	2541769820	AvCA_16200	hypothetical protein	114
495	2541771820	AvCA_36220	integrating conjugative element membrane protein, PFL_4702 family	114
279	2541770637	AvCA_24400	hypothetical protein	113
516	2541771916	AvCA_37170	hypothetical protein	111
75	2541768975	AvCA_07720	hypothetical protein	110
262	2541770456	AvCA_22610	hypothetical protein	110
549	2541771952	AvCA_37550	phage holin, lambda family	110
572	2541772211	AvCA_40180	hypothetical protein	109
96	2541769140	AvCA_09410	hypothetical protein	108
696	2541773376	AvCA_52110	hypothetical protein	108
171	2541769615	AvCA_14180	hypothetical protein	107
213	2541769978	AvCA_17750	hypothetical protein	107
258	2541770436	AvCA_22410	hypothetical protein	107
437	2541771665	AvCA_34700	hypothetical protein	107
673	2541773158	AvCA_49780	protein of unknown function (DUF3893)	107
226	2541770079	AvCA_18770	hypothetical protein	106
485	2541771807	AvCA_36100	probable addiction module antidote protein	106
444	2541771758	AvCA_35600	Transposase	105
618	2541772562	AvCA_43700	hypothetical protein	105
435	2541771653	AvCA_34580	hypothetical protein	104
246	2541770362	AvCA_21650	hypothetical protein	103
3	2541768254	AvCA_00560	hypothetical protein	102
7	2541768270	AvCA_00720	Acetyltransferase (GNAT) domain-containing protein	102
391	2541771427	AvCA_32330	DDE superfamily endonuclease	102
11	2541768301	AvCA_01030	hypothetical protein	101

175	2541769679	AvCA_14820	hypothetical protein	101
450	2541771764	AvCA_35670	lipoprotein	100
497	2541771827	AvCA_36290	hypothetical protein	99
534	2541771936	AvCA_37380	Phage tail assembly chaperone protein, E, or 41 or 14	99
537	2541771939	AvCA_37410	hypothetical protein	99
690	2541773306	AvCA_51380	hypothetical protein	99
174	2541769640	AvCA_14430	hypothetical protein	97
291	2541770709	AvCA_25110	Putative conjugal transfer nickase/helicase TraI C-term	97
408	2541771529	AvCA_33400	hypothetical protein	97
370	2541771352	AvCA_31570	CRISPR-associated protein, Cas2 family	95
116	2541769173	AvCA_09770	transposase	94
422	2541771569	AvCA_33800	hypothetical protein	94
453	2541771767	AvCA_35700	type IV secretion system protein VirB3	94
454	2541771768	AvCA_35710	type IV secretion system protein VirB2	94
575	2541772247	AvCA_40540	transposase	94
593	2541772377	AvCA_41880	transposase	94
658	2541773142	AvCA_49610	hypothetical protein	94
308	2541770827	AvCA_26330	hypothetical protein	93
318	2541770905	AvCA_27120	hypothetical protein	93
191	2541769853	AvCA_16540	transposase	92
232	2541770258	AvCA_20550	transposase	92
81	2541769002	AvCA_07970	hypothetical protein	91
626	2541772638	AvCA_44480	hypothetical protein	91
475	2541771796	AvCA_35990	DNA binding domain-containing protein, excisionase family	89
524	2541771926	AvCA_37280	hypothetical protein	89
458	2541771772	AvCA_35750	lipoprotein	88
464	2541771781	AvCA_35840	lipoprotein	87
522	2541771923	AvCA_37250	hypothetical protein	87
16	2541768441	AvCA_02440	hypothetical protein	86
32	2541768587	AvCA_03920	hypothetical protein	86
36	2541768614	AvCA_04180	hypothetical protein	86
186	2541769806	AvCA_16060	Transposase IS200 like	86
125	2541769261	AvCA_10650	hypothetical protein	85
349	2541771149	AvCA_29570	hypothetical protein	85
106	2541769155	AvCA_09580	Transposase DDE domain-containing protein	84
127	2541769280	AvCA_10840	Transposase DDE domain-containing protein	84
176	2541769698	AvCA_15010	Transposase DDE domain-containing protein	84
277	2541770591	AvCA_23940	Transposase DDE domain-containing protein	84
406	2541771523	AvCA_33330	Transposase DDE domain-containing protein	84
425	2541771584	AvCA_33950	hypothetical protein	84
472	2541771793	AvCA_35960	plasmid segregation centromere-binding protein ParG	84
488	2541771813	AvCA_36160	Transposase DDE domain-containing protein	84
680	2541773165	AvCA_49850	Transposase DDE domain-containing protein	84
312	2541770886	AvCA_26930	hypothetical protein	83
350	2541771206	AvCA_30150	hypothetical protein	83
60	2541768787	AvCA_05870	hypothetical protein	82
259	2541770441	AvCA_22460	hypothetical protein	82

445	2541771759	AvCA_35620	hypothetical protein	81
484	2541771806	AvCA_36090	hypothetical protein	81
271	2541770553	AvCA_23560	hypothetical protein	80
403	2541771497	AvCA_33070	hypothetical protein	80
28	2541768562	AvCA_03660	hypothetical protein	79
494	2541771819	AvCA_36210	integrating conjugative element protein, PFL_4701 family	79
633	2541772692	AvCA_45030	hypothetical protein	79
58	2541768772	AvCA_05720	hypothetical protein	78
256	2541770418	AvCA_22230	hypothetical protein	78
478	2541771799	AvCA_36020	Transcriptional regulator Cro/CI-like protein	78
492	2541771817	AvCA_36190	antitoxin VapB	78
506	2541771847	AvCA_36490	Integrase, catalytic domain-containing protein	78
656	2541773098	AvCA_49130	hypothetical protein	78
662	2541773146	AvCA_49650	hypothetical protein	77
257	2541770429	AvCA_22340	hypothetical protein	76
574	2541772218	AvCA_40250	hypothetical protein	76
526	2541771928	AvCA_37300	hypothetical protein	75
120	2541769183	AvCA_09870	hypothetical protein	74
135	2541769360	AvCA_11660	hypothetical protein	74
152	2541769451	AvCA_12540	hypothetical protein	74
219	2541770017	AvCA_18140	hypothetical protein	74
227	2541770094	AvCA_18920	hypothetical protein	74
442	2541771739	AvCA_35410	hypothetical protein	74
570	2541772196	AvCA_40010	hypothetical protein	74
621	2541772580	AvCA_43880	hypothetical protein	74
98	2541769143	AvCA_09450	hypothetical protein	73
354	2541771222	AvCA_30310	hypothetical protein	73
12	2541768323	AvCA_01250	hypothetical protein	72
423	2541771572	AvCA_33830	hypothetical protein	72
426	2541771586	AvCA_33970	hypothetical protein	72
692	2541773348	AvCA_51830	hypothetical protein	72
64	2541768894	AvCA_06920	hypothetical protein	71
685	2541773220	AvCA_50430	hypothetical protein	71
35	2541768613	AvCA_04170	hypothetical protein	70
51	2541768742	AvCA_05420	hypothetical protein	70
322	2541770936	AvCA_27430	hypothetical protein	70
663	2541773147	AvCA_49660	putative transcriptional regulator	70
33	2541768592	AvCA_03970	hypothetical protein	69
188	2541769850	AvCA_16500	MFS transporter, FHS family, L-fucose permease	69
561	2541772026	AvCA_38290	hypothetical protein	69
404	2541771511	AvCA_33220	hypothetical protein	68
568	2541772167	AvCA_39710	hypothetical protein	68
597	2541772385	AvCA_41960	hypothetical protein	68
623	2541772603	AvCA_44110	transposase	68
317	2541770903	AvCA_27100	hypothetical protein	67
400	2541771458	AvCA_32650	hypothetical protein	67
611	2541772511	AvCA_43190	hypothetical protein	67

46	2541768690	AvCA_04890	hypothetical protein	66
340	2541771085	AvCA_28900	hypothetical protein	66
641	2541772822	AvCA_46330	hypothetical protein	66
85	2541769017	AvCA_08120	hypothetical protein	65
240	2541770298	AvCA_20960	hypothetical protein	65
305	2541770794	AvCA_25980	hypothetical protein	65
411	2541771533	AvCA_33420	Diguanylate cyclase, GGDEF domain	65
573	2541772213	AvCA_40200	hypothetical protein	65
221	2541770032	AvCA_18300	hypothetical protein	64
304	2541770770	AvCA_25720	hypothetical protein	64
546	2541771949	AvCA_37510	hypothetical protein	64
83	2541769006	AvCA_08010	hypothetical protein	63
93	2541769108	AvCA_09090	hypothetical protein	63
208	2541769927	AvCA_17240	CRISPR-associated protein, Cas2 family	63
297	2541770746	AvCA_25470	transposase	63
329	2541771000	AvCA_28040	transposase	63
419	2541771561	AvCA_33720	hypothetical protein	63
564	2541772115	AvCA_39190	hypothetical protein	63
267	2541770494	AvCA_23010	hypothetical protein	62
559	2541772008	AvCA_38110	hypothetical protein	62
10	2541768276	AvCA_00780	hypothetical protein	61
20	2541768465	AvCA_02680	hypothetical protein	61
117	2541769174	AvCA_09780	transposase, IS4 family	61
343	2541771098	AvCA_29040	hypothetical protein	61
615	2541772535	AvCA_43430	hypothetical protein	61
637	2541772782	AvCA_45970	hypothetical protein	61
82	2541769004	AvCA_07990	hypothetical protein	60
179	2541769714	AvCA_15170	hypothetical protein	60
26	2541768544	AvCA_03480	hypothetical protein	59
80	2541768998	AvCA_07930	hypothetical protein	59
286	2541770664	AvCA_24690	hypothetical protein	59
388	2541771423	AvCA_32290	hypothetical protein	59
557	2541771991	AvCA_37940	hypothetical protein	59
571	2541772202	AvCA_40080	hypothetical protein	59
614	2541772520	AvCA_43280	hypothetical protein	59
17	2541768459	AvCA_02620	hypothetical protein	58
181	2541769738	AvCA_15410	hypothetical protein	58
237	2541770280	AvCA_20780	hypothetical protein	58
616	2541772549	AvCA_43570	hypothetical protein	58
41	2541768629	AvCA_04310	hypothetical protein	57
136	2541769365	AvCA_11710	hypothetical protein	57
209	2541769948	AvCA_17450	hypothetical protein	57
321	2541770934	AvCA_27410	hypothetical protein	57
511	2541771901	AvCA_37030	hypothetical protein	57
334	2541771030	AvCA_28340	hypothetical protein	56
627	2541772646	AvCA_44560	hypothetical protein	56
643	2541772845	AvCA_46570	hypothetical protein	56

34	2541768599	AvCA_04040	hypothetical protein	55
59	2541768781	AvCA_05810	hypothetical protein	55
121	2541769234	AvCA_10370	hypothetical protein	55
211	2541769961	AvCA_17580	hypothetical protein	55
265	2541770485	AvCA_22920	hypothetical protein	55
289	2541770676	AvCA_24800	hypothetical protein	55
118	2541769175	AvCA_09790	transposase	54
192	2541769856	AvCA_16570	hypothetical protein	54
207	2541769926	AvCA_17230	hypothetical protein	54
230	2541770146	AvCA_19390	non-hypothetical protein	54
338	2541771069	AvCA_28730	hypothetical protein	54
399	2541771456	AvCA_32630	hypothetical protein	54
581	2541772292	AvCA_40990	hypothetical protein	54
622	2541772602	AvCA_44100	hypothetical protein	54
54	2541768755	AvCA_05550	hypothetical protein	53
70	2541768923	AvCA_07220	hypothetical protein	53
377	2541771359	AvCA_31640	hypothetical protein	53
443	2541771741	AvCA_35430	hypothetical protein	53
565	2541772125	AvCA_39290	hypothetical protein	53
686	2541773239	AvCA_50620	hypothetical protein	53
238	2541770290	AvCA_20880	hypothetical protein	52
314	2541770889	AvCA_26960	hypothetical protein	52
521	2541771922	AvCA_37240	hypothetical protein	52
311	2541770878	AvCA_26850	hypothetical protein	51
598	2541772390	AvCA_42020	proteic killer suppression protein	51
682	2541773167	AvCA_49870	hypothetical protein	51
50	2541768723	AvCA_05230	hypothetical protein	50
72	2541768944	AvCA_07430	hypothetical protein	50
91	2541769090	AvCA_08900	hypothetical protein	50
182	2541769740	AvCA_15430	hypothetical protein	50
420	2541771563	AvCA_33740	putative transposase	50
657	2541773124	AvCA_49430	hypothetical protein	50
529	2541771931	AvCA_37330	protein of unknown function (DUF4160)	49
619	2541772574	AvCA_43820	hypothetical protein	49
648	2541772957	AvCA_47680	hypothetical protein	49
1	2541768229	AvCA_00310	hypothetical protein	48
228	2541770107	AvCA_19050	hypothetical protein	48
567	2541772154	AvCA_39580	hypothetical protein	48
128	2541769295	AvCA_10990	hypothetical protein	47
150	2541769428	AvCA_12310	hypothetical protein	47
193	2541769870	AvCA_16710	hypothetical protein	47
203	2541769909	AvCA_17070	hypothetical protein	47
261	2541770449	AvCA_22540	hypothetical protein	47
268	2541770504	AvCA_23110	hypothetical protein	47
284	2541770656	AvCA_24610	transposase, IS5 family	47
348	2541771141	AvCA_29480	hypothetical protein	47
440	2541771686	AvCA_34910	hypothetical protein	47

684	2541773181	AvCA_50010	hypothetical protein	47
53	2541768752	AvCA_05520	hypothetical protein	46
280	2541770642	AvCA_24450	hypothetical protein	46
61	2541768824	AvCA_06210	hypothetical protein	45
89	2541769055	AvCA_08500	hypothetical protein	45
239	2541770292	AvCA_20900	hypothetical protein	45
316	2541770899	AvCA_27060	hypothetical protein	45
431	2541771603	AvCA_34120	hypothetical protein	45
479	2541771800	AvCA_36030	hypothetical protein	45
675	2541773160	AvCA_49800	hypothetical protein	45
6	2541768269	AvCA_00710	hypothetical protein	44
167	2541769572	AvCA_13800	hypothetical protein	44
254	2541770406	AvCA_22110	hypothetical protein	44
432	2541771611	AvCA_34200	hypothetical protein	44
23	2541768523	AvCA_03270	hypothetical protein	43
24	2541768530	AvCA_03340	hypothetical protein	43
86	2541769024	AvCA_08190	hypothetical protein	43
156	2541769543	AvCA_13460	hypothetical protein	43
210	2541769950	AvCA_17470	hypothetical protein	43
243	2541770317	AvCA_21150	hypothetical protein	43
359	2541771290	AvCA_30950	hypothetical protein	43
498	2541771829	AvCA_36310	hypothetical protein	43
560	2541772009	AvCA_38120	hypothetical protein	43
92	2541769106	AvCA_09070	hypothetical protein	42
122	2541769239	AvCA_10420	hypothetical protein	42
180	2541769733	AvCA_15360	hypothetical protein	42
327	2541770976	AvCA_27800	hypothetical protein	42
533	2541771935	AvCA_37370	Phage P2 GpE	42
554	2541771963	AvCA_37660	hypothetical protein	42
617	2541772560	AvCA_43680	hypothetical protein	42
95	2541769119	AvCA_09200	hypothetical protein	41
214	2541769992	AvCA_17890	hypothetical protein	41
260	2541770444	AvCA_22490	hypothetical protein	41
381	2541771396	AvCA_32010	hypothetical protein	41
393	2541771439	AvCA_32450	hypothetical protein	41
584	2541772354	AvCA_41630	hypothetical protein	41
599	2541772396	AvCA_42080	hypothetical protein	41
44	2541768681	AvCA_04800	hypothetical protein	40
56	2541768770	AvCA_05700	hypothetical protein	40
74	2541768974	AvCA_07710	hypothetical protein	40
270	2541770520	AvCA_23270	hypothetical protein	40
302	2541770757	AvCA_25590	hypothetical protein	40
394	2541771443	AvCA_32490	hypothetical protein	40
417	2541771551	AvCA_33620	hypothetical protein	40
438	2541771674	AvCA_34790	hypothetical protein	40
578	2541772273	AvCA_40800	hypothetical protein	40
670	2541773154	AvCA_49740	hypothetical protein	40

132	2541769327	AvCA_11330	hypothetical protein	39
204	2541769916	AvCA_17130	hypothetical protein	39
293	2541770726	AvCA_25260	hypothetical protein	39
433	2541771647	AvCA_34520	hypothetical protein	39
706	2541773411	AvCA_52490	hypothetical protein	39
325	2541770955	AvCA_27620	hypothetical protein	38
326	2541770970	AvCA_27740	hypothetical protein	38
330	2541771001	AvCA_28050	transposase	38
580	2541772288	AvCA_40950	hypothetical protein	38
57	2541768771	AvCA_05710	hypothetical protein	37
65	2541768908	AvCA_07060	glyceraldehyde-3-phosphate dehydrogenase (NADP+)	37
133	2541769342	AvCA_11480	hypothetical protein	37
168	2541769585	AvCA_13930	hypothetical protein	37
216	2541770009	AvCA_18060	hypothetical protein	37
285	2541770662	AvCA_24670	hypothetical protein	37
607	2541772507	AvCA_43150	hypothetical protein	37
612	2541772514	AvCA_43220	hypothetical protein	37
624	2541772604	AvCA_44120	putative transposase	37
73	2541768964	AvCA_07610	hypothetical protein	36
90	2541769089	AvCA_08890	hypothetical protein	36
212	2541769976	AvCA_17730	hypothetical protein	36
223	2541770043	AvCA_18410	hypothetical protein	36
337	2541771066	AvCA_28700	hypothetical protein	36
351	2541771209	AvCA_30180	hypothetical protein	36
392	2541771432	AvCA_32380	hypothetical protein	36
424	2541771582	AvCA_33930	hypothetical protein	36
439	2541771677	AvCA_34820	hypothetical protein	36
646	2541772864	AvCA_46760	hypothetical protein	36
683	2541773180	AvCA_50000	hypothetical protein	36
52	2541768744	AvCA_05440	hypothetical protein	35
63	2541768875	AvCA_06720	hypothetical protein	35
130	2541769302	AvCA_11060	hypothetical protein	35
148	2541769423	AvCA_12260	hypothetical protein	35
231	2541770234	AvCA_20320	hypothetical protein	35
234	2541770261	AvCA_20580	hypothetical protein	35
253	2541770397	AvCA_22020	hypothetical protein	35
263	2541770474	AvCA_22790	hypothetical protein	35
313	2541770888	AvCA_26950	hypothetical protein	35
319	2541770907	AvCA_27140	hypothetical protein	35
333	2541771028	AvCA_28320	hypothetical protein	35
416	2541771549	AvCA_33600	hypothetical protein	35
427	2541771590	AvCA_34010	hypothetical protein	35
620	2541772576	AvCA_43840	hypothetical protein	35
21	2541768485	AvCA_02890	hypothetical protein	34
27	2541768552	AvCA_03560	hypothetical protein	34
45	2541768682	AvCA_04810	hypothetical protein	34
149	2541769427	AvCA_12300	hypothetical protein	34

194	2541769874	AvCA_16750	hypothetical protein	34
269	2541770517	AvCA_23240	hypothetical protein	34
288	2541770674	AvCA_24780	hypothetical protein	34
309	2541770831	AvCA_26370	hypothetical protein	34
384	2541771414	AvCA_32200	hypothetical protein	34
387	2541771422	AvCA_32280	hypothetical protein	34
436	2541771657	AvCA_34620	hypothetical protein	34
594	2541772379	AvCA_41900	hypothetical protein	34
645	2541772861	AvCA_46730	hypothetical protein	34
22	2541768519	AvCA_03230	hypothetical protein	33
38	2541768618	AvCA_04210	hypothetical protein	33
55	2541768756	AvCA_05560	hypothetical protein	33
172	2541769617	AvCA_14200	hypothetical protein	33
199	2541769895	AvCA_16930	hypothetical protein	33
215	2541770006	AvCA_18030	hypothetical protein	33
275	2541770588	AvCA_23910	hypothetical protein	33
323	2541770944	AvCA_27510	hypothetical protein	33
352	2541771210	AvCA_30190	hypothetical protein	33
397	2541771453	AvCA_32600	hypothetical protein	33
515	2541771915	AvCA_37160	hypothetical protein	33
519	2541771920	AvCA_37220	hypothetical protein	33
19	2541768464	AvCA_02670	hypothetical protein	32
362	2541771304	AvCA_31090	hypothetical protein	32
379	2541771361	AvCA_31660	hypothetical protein	32
383	2541771401	AvCA_32060	hypothetical protein	32
421	2541771565	AvCA_33760	hypothetical protein	32
441	2541771723	AvCA_35260	hypothetical protein	32
510	2541771876	AvCA_36770	hypothetical protein	32
586	2541772361	AvCA_41700	hypothetical protein	32
595	2541772380	AvCA_41910	hypothetical protein	32
647	2541772898	AvCA_47090	hypothetical protein	32
303	2541770768	AvCA_25700	hypothetical protein	31
307	2541770811	AvCA_26170	hypothetical protein	31
339	2541771075	AvCA_28800	hypothetical protein	31
630	2541772665	AvCA_44750	hypothetical protein	31
631	2541772677	AvCA_44870	hypothetical protein	31
15	2541768413	AvCA_02160	hypothetical protein	30
42	2541768640	AvCA_04420	hypothetical protein	30
47	2541768694	AvCA_04930	hypothetical protein	30
49	2541768721	AvCA_05210	hypothetical protein	30
173	2541769637	AvCA_14400	hypothetical protein	30
224	2541770051	AvCA_18490	hypothetical protein	30
247	2541770364	AvCA_21670	hypothetical protein	30
273	2541770572	AvCA_23760	hypothetical protein	30
274	2541770586	AvCA_23900	hypothetical protein	30
320	2541770922	AvCA_27290	hypothetical protein	30
380	2541771388	AvCA_31930	HigB_toxin, RelE-like toxic component of a toxin-antitoxin system	30

579	2541772285	AvCA_40920	hypothetical protein	30
585	2541772359	AvCA_41680	hypothetical protein	30
655	2541773068	AvCA_48820	hypothetical protein	30
4	2541768266	AvCA_00680	hypothetical protein	29
14	2541768412	AvCA_02150	hypothetical protein	29
48	2541768720	AvCA_05200	hypothetical protein	29
129	2541769299	AvCA_11030	hypothetical protein	29
139	2541769383	AvCA_11900	hypothetical protein	29
145	2541769413	AvCA_12160	hypothetical protein	29
183	2541769756	AvCA_15560	hypothetical protein	29
217	2541770011	AvCA_18080	hypothetical protein	29
218	2541770016	AvCA_18130	hypothetical protein	29
266	2541770489	AvCA_22960	hypothetical protein	29
272	2541770558	AvCA_23610	hypothetical protein	29
301	2541770752	AvCA_25540	hypothetical protein	29
358	2541771278	AvCA_30830	hypothetical protein	29
613	2541772519	AvCA_43270	hypothetical protein	29
674	2541773159	AvCA_49790	hypothetical protein	29
679	2541773164	AvCA_49840	hypothetical protein	29
8	2541768271	AvCA_00730	hypothetical protein	28
79	2541768995	AvCA_07900	hypothetical protein	28
84	2541769016	AvCA_08110	hypothetical protein	28
154	2541769528	AvCA_13310	hypothetical protein	28
178	2541769707	AvCA_15100	hypothetical protein	28
290	2541770691	AvCA_24950	hypothetical protein	28
336	2541771064	AvCA_28680	hypothetical protein	28
389	2541771424	AvCA_32300	hypothetical protein	28
395	2541771451	AvCA_32580	transposase, IS204/IS1001/IS1096/IS1165	28
430	2541771600	AvCA_34110	hypothetical protein	28
468	2541771788	AvCA_35910	hypothetical protein	28
513	2541771913	AvCA_37140	hypothetical protein	28
644	2541772849	AvCA_46610	hypothetical protein	28
18	2541768460	AvCA_02630	hypothetical protein	27
119	2541769178	AvCA_09820	hypothetical protein	27
123	2541769242	AvCA_10450	hypothetical protein	27
124	2541769244	AvCA_10470	hypothetical protein	27
151	2541769433	AvCA_12360	hypothetical protein	27
508	2541771851	AvCA_36530	hypothetical protein	27
562	2541772027	AvCA_38300	hypothetical protein	27
691	2541773321	AvCA_51540	hypothetical protein	27
29	2541768569	AvCA_03730	hypothetical protein	26
30	2541768582	AvCA_03860	hypothetical protein	26
87	2541769031	AvCA_08260	hypothetical protein	26
94	2541769116	AvCA_09170	hypothetical protein	26
137	2541769366	AvCA_11720	hypothetical protein	26
142	2541769405	AvCA_12100	hypothetical protein	26
196	2541769887	AvCA_16860	hypothetical protein	26

225	2541770064	AvCA_18620	hypothetical protein	26
306	2541770803	AvCA_26080	hypothetical protein	26
335	2541771063	AvCA_28670	hypothetical protein	26
344	2541771119	AvCA_29250	hypothetical protein	26
347	2541771135	AvCA_29420	hypothetical protein	26
365	2541771341	AvCA_31470	hypothetical protein	26
481	2541771803	AvCA_36060	hypothetical protein	26
500	2541771831	AvCA_36330	transposase	26
556	2541771988	AvCA_37910	hypothetical protein	26
577	2541772267	AvCA_40740	hypothetical protein	26
601	2541772413	AvCA_42250	hypothetical protein	26
702	2541773401	AvCA_52380	hypothetical protein	26
13	2541768406	AvCA_02090	hypothetical protein	25
40	2541768627	AvCA_04290	hypothetical protein	25
170	2541769607	AvCA_14100	hypothetical protein	25
324	2541770949	AvCA_27560	hypothetical protein	25
328	2541770989	AvCA_27930	hypothetical protein	25
366	2541771348	AvCA_31540	hypothetical protein	25
418	2541771552	AvCA_33630	hypothetical protein	25
563	2541772071	AvCA_38760	hypothetical protein	25
576	2541772257	AvCA_40640	hypothetical protein	25
649	2541772988	AvCA_47990	hypothetical protein	25
650	2541773020	AvCA_48320	hypothetical protein	25
62	2541768863	AvCA_06600	hypothetical protein	24
71	2541768934	AvCA_07330	hypothetical protein	24
198	2541769894	AvCA_16920	hypothetical protein	24
233	2541770259	AvCA_20560	hypothetical protein	24
242	2541770306	AvCA_21040	hypothetical protein	24
332	2541771026	AvCA_28300	hypothetical protein	24
342	2541771087	AvCA_28930	hypothetical protein	24
364	2541771340	AvCA_31460	hypothetical protein	24
555	2541771972	AvCA_37750	hypothetical protein	24
605	2541772505	AvCA_43130	hypothetical protein	24
653	2541773066	AvCA_48800	hypothetical protein	24
669	2541773153	AvCA_49730	hypothetical protein	24

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