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# A novel bleb-dependent polysaccharide export system in nitrogen-fixing *Azotobacter vinelandii* subjected to low nitrogen gas levels

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**Summary.** The alginate biofilm-producing bacterium *Azotobacter vinelandii* aerobically fixes nitrogen by oxygen-sensitive nitrogenases. Here we investigated the bacterial response to nitrogen/oxygen gas mixtures. *A. vinelandii* cells were cultured in nitrogen-free minimal media containing gas mixtures differing in their ratios of nitrogen and oxygen. The bacteria did not grow at oxygen concentrations >75 % but grew well in the presence of 5 % nitrogen/25 % oxygen. Growth of wild-type and alginate-deficient strains when cultured with 50 % oxygen did not differ substantially, indicating that alginate is not required for the protection of nitrogenases from oxygen damage. In response to decreasing nitrogen levels, *A. vinelandii* produced greater amounts of alginate, accompanied by the formation of blebs on the cell surface. The encystment of vegetative cells occurred in tandem with the release of blebs and the development of a multilayered exine. Immunoelectron microscopy using anti alginate-antibody revealed that the blebs contained alginate molecules. By contrast, alginate-deficient mutants could not form blebs. Taken together, our data provide evidence for a novel bleb-dependent polysaccharide export system in *A. vinelandii* that is activated in response to low nitrogen gas levels. [Int Microbiol 2013; 16(1):35-44]

**Keywords:** *Azotobacter vinelandii* · alginate · nitrogen stress · outer membrane vesicles · bleb-dependent export

## Introduction

*Azotobacter vinelandii* is a member of the Gamma-proteobacteria that under oxic conditions fixes atmospheric nitrogen by converting it into ammonia [8]. Nitrogen fixation requires the expression of molybdenum-, vanadium-,

and iron-nitrogenases [30] and large amounts of ATP, which in turn implies a role for aerobic respiration. Since the three nitrogenases are sensitive to oxygen, bacterial cells protect them from oxygen damage through the removal of intracellular oxygen by an unknown mechanism and by cell-surface repression of the influx of oxygen from the outside. In the latter, two possible mechanisms have been suggested: respiratory protection and the development of an alginate biofilm that acts as a barrier [2,24].

Cell-surface proteins are responsible for respiratory protection by consuming oxygen as the final electron acceptor [22]. Bacterial genome sequencing and biochemical experimental data have revealed that four NADH-

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ubiquinone oxidoreductases, five terminal oxidases, and two ATP synthases confer respiratory protection [28]. Their expression is positively or negatively controlled by the oxygen-responsive transcriptional regulator CydR.

Alginate is a linear polysaccharide consisting of two monosaccharides,  $\beta$ -D-mannuronate and its C-5 epimer  $\alpha$ -L-guluronate [6]. It protects nitrogenases from oxygen damage by inhibiting oxygen uptake under phosphate-limited conditions [24]. *A. vinelandii* cells produce alginate as a component of the extracellular biofilm. In addition to *A. vinelandii*, pseudomonads such as *Pseudomonas aeruginosa* and *P. syringae* secrete alginate biofilms [6,14]. In both azotobacters and pseudomonads the synthesis and secretion of alginate is mediated by *algA*, *algD*, *algE*, *algF*, *algG*, *algI*, *algJ*, *algK*, *algL*, *algX*, *alg8*, and *alg44*. These genes are assembled into a single cluster and their expression is closely regulated by several factors, such as the sigma factor AlgU [6,23].

In the case of *A. vinelandii*, the molecular weight, production level, and mannuronate/guluronate ratio of alginate vary depending on the dissolved oxygen tension [21]. Diverse mannuronate C-5-epimerases specific for *A. vinelandii* cells have been implicated in the increase in guluronate that, together with divalent cations, is essential for the formation of an alginate barrier [29]. GDP-mannose 6-dehydrogenase (AlgD) catalyzes the conversion of GDP-mannose to GDP-mannuronic acid and plays a crucial role in alginate synthesis [3], which occurs during the stationary phase of the growth cycle. The expression of *algD* and thus alginate formation are controlled by a two-component system consisting of GacS-GacA, which regulates the transcription level of the stationary phase-specific sigma factor RpoS [7]. Thus, the disruption of *algD* either indirectly, by alterations in one or both of these components, or directly, e.g., by alterations in the gene itself, prohibits alginate production by *A. vinelandii* [3].

The bacterial response to oxygen has been well studied with respect to the protection of nitrogenases from oxygen damage and during phosphate limitation [2,7,18,21,23], whereas little is known about the bacterial response to disturbances in nitrogen levels under normal phosphate conditions. In this study, the effects of nitrogen/oxygen levels on bacterial growth, alginate production, and cell structure were examined in wild-type *A. vinelandii* strain ATCC 12837 cells and in the mutant, which is deficient in alginate production through the insertion of a tetracycline-resistance gene into *algD*.

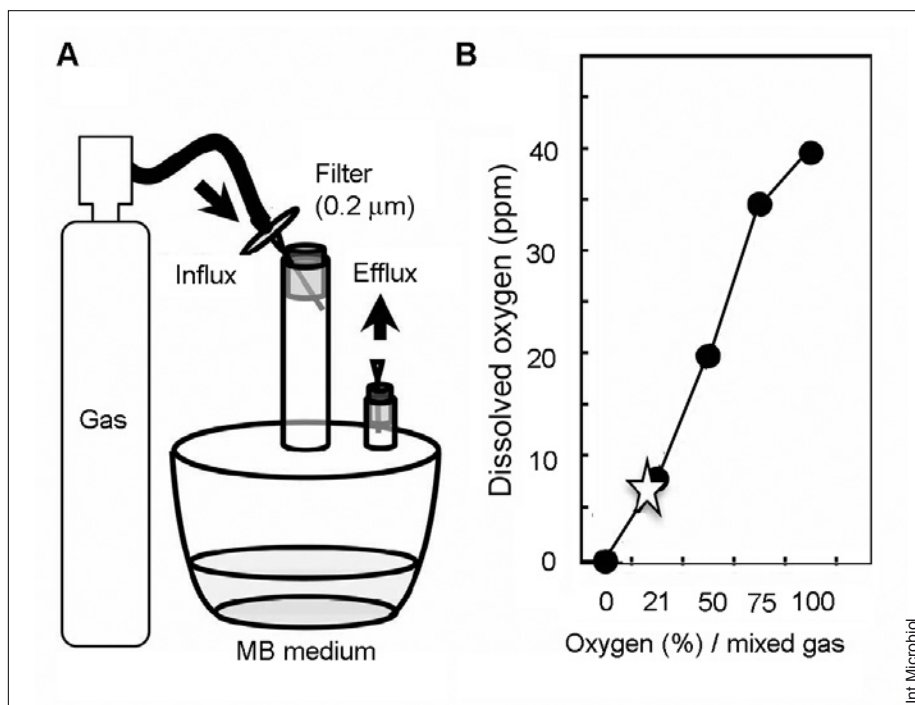
## Materials and methods

**Bacterial growth.** *Azotobacter vinelandii* strain ATCC 12837 (NBRC 13581 purchased from the National Institute of Technology and Evaluation, Japan) was aerobically precultured at 30 °C in nitrogen-free modified Burk's (MB) medium [20] consisting of 20 mg sucrose/ml, 0.2 mg NaCl/ml, 0.05 mg CaSO<sub>4</sub>·2H<sub>2</sub>O/ml, 0.2 mg MgSO<sub>4</sub>·7H<sub>2</sub>O/ml, 2.9  $\mu$ g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O/ml, 27  $\mu$ g FeSO<sub>4</sub>·7H<sub>2</sub>O/ml, 0.66 mg K<sub>2</sub>HPO<sub>4</sub>/ml, and 0.16 mg KH<sub>2</sub>PO<sub>4</sub>/ml. The bacterial cells were grown at 30 °C with shaking at 123 rpm in 50 ml of MB medium in 500-ml airtight flasks, except during the 5-min injection of the filter-sterilized gas mixture at a pressure of 0.05 MPa every 8 h, as described below (Fig. 1A). Gas injection was evaluated using a single-channel oxygen meter (Fibox 3, Presens) to determine the dissolved oxygen concentration (Fig. 1B).

**Cultivation under various gas-mixture conditions.** The gas mixtures were purchased from Imamura Oxygen, Kyoto, Japan. The involvement of alginate in nitrogen fixation was examined in wild-type *A. vinelandii* strain ATCC 12837 and in mutant cells carrying a disrupted *algD* gene. Both were grown on nitrogen (yeast extract)-free or supplemented MB agar plates under atmospheric air conditions (approximately 78 % nitrogen, 21 % oxygen, and trace amounts of other gases). Bacterial growth in response to the various gas mixtures was determined by cultivating the wild-type and mutant cells in MB liquid media in the presence of gas mixtures containing various ratios of nitrogen and oxygen (Fig. 1) as follows: The cells were cultivated under hermetic conditions. Every 8 h, they were exposed for 5 min to artificial air containing 79 % nitrogen and 21 % oxygen (N79/O21), N100, 50 % nitrogen/25 % oxygen/25% helium (N50/O25), N50/O50, N25/O75, or O100.

**Plasmid construction.** The *algD* gene [3] was cloned from the genomic DNA of *A. vinelandii* cells using the DNeasy blood & tissue kit (Qiagen, Tokyo, Japan). To construct the *algD* disruptant, *algD* (1.3 kb) was PCR-amplified using KOD-Plus polymerase (Toyobo, Osaka, Japan), the *A. vinelandii* genomic DNA as template, and two synthetic oligonucleotide primers (forward, 5'-GGGAATTCTTTTCGGACTGGGCTATGTAGG-3'; reverse, 5'-GGGAATTCACCAGCAGATGCCTTCGTC-3') (Hokkaido System Science, Sapporo, Japan) containing an *EcoRI* site at their 5' ends. After amplification, the PCR product was ligated using Ligation High (Toyobo) with *HincII*-digested pUC119 (Takara Bio, Otsu, Japan). The resultant plasmid was designated pUC119-*algD*. The tetracycline-resistance gene (1.9 kb) was PCR-amplified using KOD-Plus polymerase, the plasmid pACYC184 (Nippon Gene, Tokyo, Japan) as template, and two synthetic oligonucleotide primers (forward, 5'-GGATTCTCATGTTTGACAGCTTATCATCGA-3'; reverse, 5'-CCCTACCGACAGCGGTGCGACTGTTGTA-3'). The amplified fragment was ligated into *AatI*-digested pUC119-*algD* using Ligation High, taking advantage of the unique *AatI* restriction site present in the middle of *algD*. The resultant plasmid was designated pUC119-*algD*::Tet<sup>r</sup>. After digestion of the plasmid with *EcoRI*, the fragment containing *algD*::Tet<sup>r</sup> was isolated and ligated with the *EcoRI*-digested plasmid pKTY320 [13] containing the ampicillin-resistance gene, yielding pKTY320-*algD*::Tet<sup>r</sup>. *E. coli* strain DH5 $\alpha$  cells were transformed with pKTY320-*algD*::Tet<sup>r</sup>. The correct sequence of the resultant plasmids was confirmed by DNA sequencing [27]. DNA manipulations were performed as described previously [26].

**Transformation of *Azotobacter vinelandii*.** *A. vinelandii* cells were transformed according to the method of Page and Sadoff [19]. Briefly, the cells were oxically grown at 30 °C in MB medium with



**Fig. 1.** *Azotobacter vinelandii* culture system. (A) Injection of the gas mixture into the culture flask. (B) Dissolved oxygen in the medium of cultures exposed to N100, N75/O25, N50/O50, N25/O75, or O100 was measured using an oxygen meter. The asterisk indicates the dissolved oxygen in medium exposed to atmospheric air.

10 mg glucose /ml (instead of 20 mg sucrose/ml) until they reached exponential phase (OD at 600 nm, 0.5). Fifty microliters of the *A. vinelandii* cell culture was then transferred to sterilized filter paper placed on an antibiotic-free MB plate solidified with agar and 5  $\mu$ l (1.5  $\mu$ g) of plasmid pKTY320-*algD::Tet<sup>r</sup>* was added. After 24 h of incubation at 30 °C, the bacterial cells on the filter paper were recovered in MB buffer consisting of 0.05 mg CaSO<sub>4</sub>·2H<sub>2</sub>O/ml, 0.2 mg MgSO<sub>4</sub>·7H<sub>2</sub>O/ml, 2.9  $\mu$ g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O/ml, 27  $\mu$ g FeSO<sub>4</sub>·7H<sub>2</sub>O/ml, 0.66 mg K<sub>2</sub>HPO<sub>4</sub>/ml, and 0.16 mg KH<sub>2</sub>PO<sub>4</sub>/ml and inoculated onto a tetracycline-containing MB plate, followed by incubation at 30 °C for 24 h. Tetracycline-resistant and ampicillin-sensitive colonies were isolated and designated as disruptant cells. Gene disruption in the constructed strain was confirmed by genomic PCR and DNA sequencing.

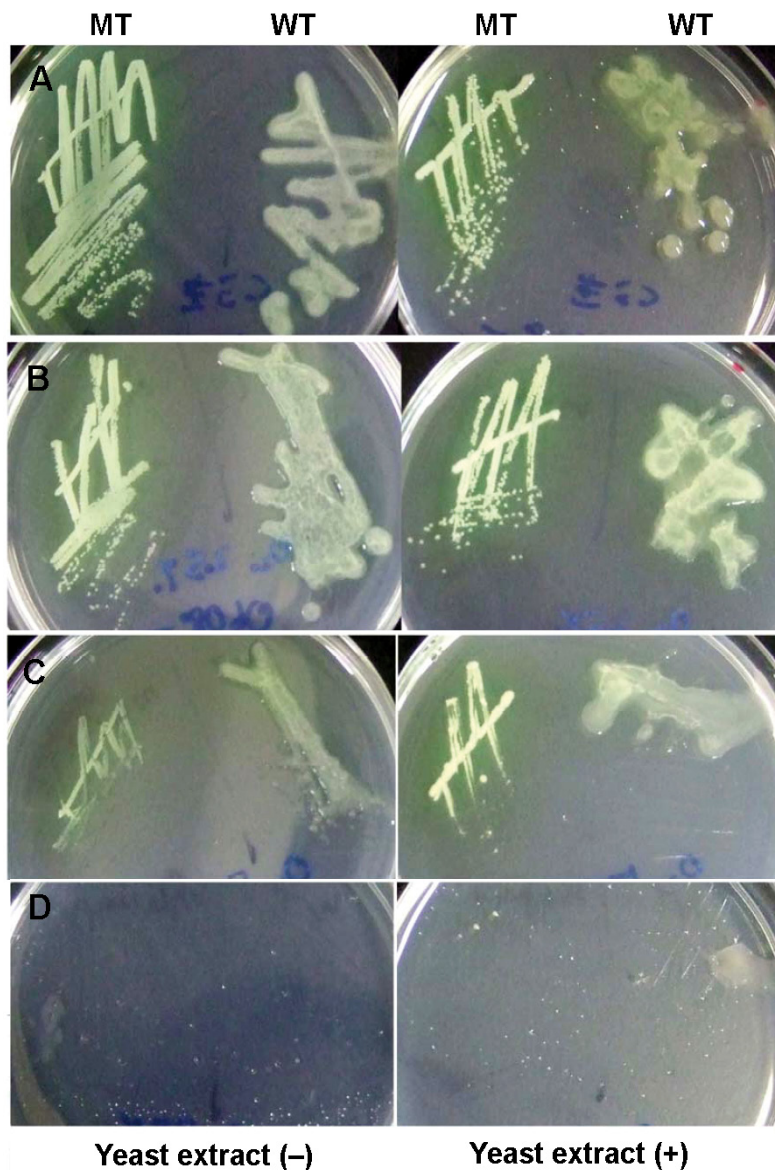
**Alginate assay.** Alginate was extracted from the bacterial culture (300  $\mu$ l) with 30  $\mu$ l of 1 M NaCl and 12  $\mu$ l of 0.5 M ethylenediaminetetraacetic acid, as described previously [24]. Bacterial cells were removed by centrifugation at 15,000  $\times$ g at 4 °C for 5 min and the resultant supernatant (280  $\mu$ l) was mixed with 900  $\mu$ l of ice-cold isopropanol at 4 °C for 10 min. The precipitated alginates were collected in a centrifugation step (15,000  $\times$ g at 4 °C for 5 min), washed with ice-cold 70 % ethanol, and dried at 25 °C under vacuum. The dried alginates were then dissolved at 4 °C for 24 h in 1 ml of distilled water. Alginate content was determined by measuring the increase in absorbance at 235 nm, indicating the detection of a double bond in the alginate degradation products formed in a reaction catalyzed by *Sphingomonas* sp. strain A1 alginate lyase A1-I, purified from recombinant *Escherichia coli* cells, as previously described [31]. Briefly, 50  $\mu$ l of 1 M Tris-HCl (pH 7.0) was added

to the diluted alginate solution (1 ml) and the absorbance was measured (preAbs<sub>235</sub>). A 500- $\mu$ l aliquot of the alginate solution was then incubated at 37 °C for 3 h with 5  $\mu$ l of 18.8  $\mu$ g A1-I/ml, and the absorbance was again measured (postAbs<sub>235</sub>). A calibration curve was obtained by measuring the increase (postAbs<sub>235</sub> – preAbs<sub>235</sub>) in the absorbance at 235 nm, using sodium alginate from the kelp *Eisenia bicyclis* (Nacalai Tesque, Kyoto, Japan) as the standard.

**Electron microscopy.** A 1-ml aliquot of *A. vinelandii* cells grown in MB medium under various mixed gases conditions until stationary phase was pre-treated with an equal volume of fixative [4 % paraformaldehyde and 4 % glutaraldehyde in 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), containing 100 mM NaCl and 2 mM CaCl<sub>2</sub> (HEPES)] at 25 °C for 1 h. After centrifugation, the bacterial cells were fixed at 4 °C for 24 h with 2 % glutaraldehyde in HEPES and washed at 4 °C with HEPES. Thin sections were prepared and analyzed at Tokai Electron Microscopy Analysis Co. (Nagoya, Japan) as follows: The fixed cells were treated at 4 °C for 2 h with 2 % OsO<sub>4</sub> in HEPES, dehydrated in an ethanol series (30 %, 50 %, 70 %, 90 %, and 100 %, 10 min each), treated twice with propylene oxide at 25 °C for 20 min, and then embedded at 60 °C for 48 h in Quetol-812 (Nissin EM, Tokyo, Japan). The resultant samples were cut with a diamond knife on a 2088 Ultratome V (LKB, Bromma, Sweden) into sections of 65–70 nm thickness, stained at 25 °C with 2 % uranyl acetate for 15 min followed by lead citrate for 3 min, and examined using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

For immunoelectron microscopy, the bacterial cells were fixed at 4 °C for 1 h by mixing the culture (1 ml) with an equal volume of fixative





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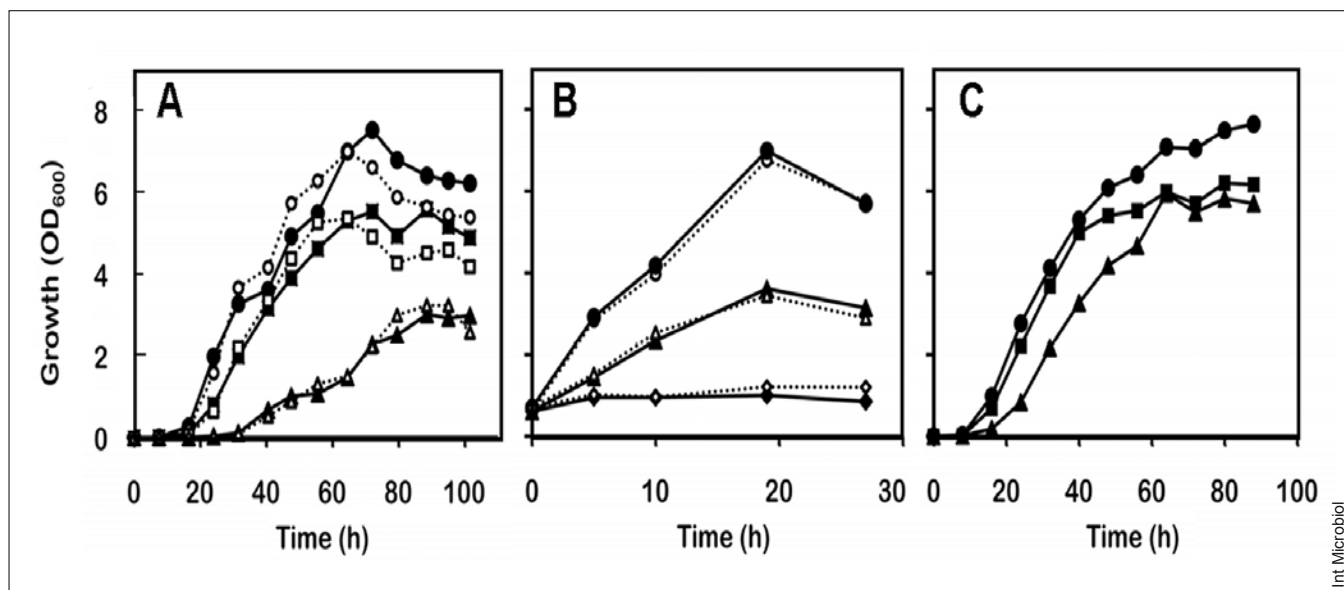
**Fig. 2.** *Azotobacter vinelandii* exposed to a gas mixture. The bacterial cells were grown on plates containing modified Burk's medium (MB) and exposed to various gas mixtures. (A) Atmospheric air. (B) N75/O25. (C) N50/O50. (D) N25/O75. WT and MT indicate the wild-type parental strain and alginate-deficient mutant, respectively. Plates on the left contained no nitrogen source, those on the right contained yeast extract.

(4 % paraformaldehyde and 0.05 % glutaraldehyde in 0.1 M phosphate buffer [pH 7.4]). After centrifugation, the bacterial cells were washed with 0.1 M phosphate buffer (pH 7.4), dehydrated at 4 °C in an ethanol series (50–70 %, 15 min each), and then embedded at 50 °C for 24 h in LR-White resin (London Resin Co., London, UK). The resultant samples were cut with a diamond knife on a 2088 Ultratome V (LKB) into 70-nm thick sections. After blocking at 25 °C for 30 min with 1% bovine serum albumin and 1.5 % goat serum in phosphate saline, the sections were incubated at 25 °C overnight with anti-alginate antibodies [4] (kindly supplied by Dr. Kazuo Okuda, Kochi University, Japan), washed with phosphate saline containing 1 % bovine serum albumin, and then incubated at 25 °C for 1 h with 10 nm colloidal gold-conjugated goat anti-rabbit IgG (BBI solutions, Cardiff, UK). The immunolabeled sections were washed with phosphate saline, fixed at 25 °C for 5 min with 2 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and stained at 25 °C with 2 % uranyl acetate for 15 min and lead citrate for 3 min. They were

then examined using a JEM-1200EX transmission electron microscope (JEOL) at 80 kV.

## Results

**Alginate unessential for protection from oxygen damage.** *Azotobacter vinelandii* cells produce alginate at the stationary growth phase [7] but not during exponential growth [11]. Wild-type cells at the stationary growth phase under atmospheric air conditions produced 0.215 mg alginate/ml, as measured in the culture broth, whereas alginate production by mutant cells carry-



**Fig. 3.** *Azotobacter vinelandii* growth profile. (A) Bacteria grown in nitrogen-free MB medium and exposed to various gas mixtures. Closed circles, wild-type strain under atmospheric air conditions; open circles, alginate-deficient mutant under atmospheric air conditions; closed squares, wild-type strain exposed to N75/O25; open squares, mutant exposed to N75/O25; closed triangles, wild-type strain exposed to N50/O50; open triangles, mutant exposed to N50/O50. (B) Gas stress of cells in the late-exponential growth phase. Closed circles, wild-type strain under atmospheric air conditions; open circles, alginate-deficient mutant under atmospheric air conditions; closed triangles, wild-type strain exposed to N50/O50; open triangles, mutant exposed to N50/O50; closed rhombus, wild-type strain exposed to N25/O75; open rhombus, mutant exposed to N25/O75. (C) The effect of nitrogen levels on bacterial growth. Wild-type cells were grown under various mixed gases conditions. Closed circles, N75/O25; closed squares, N50/O25; closed triangles, N5/O25.

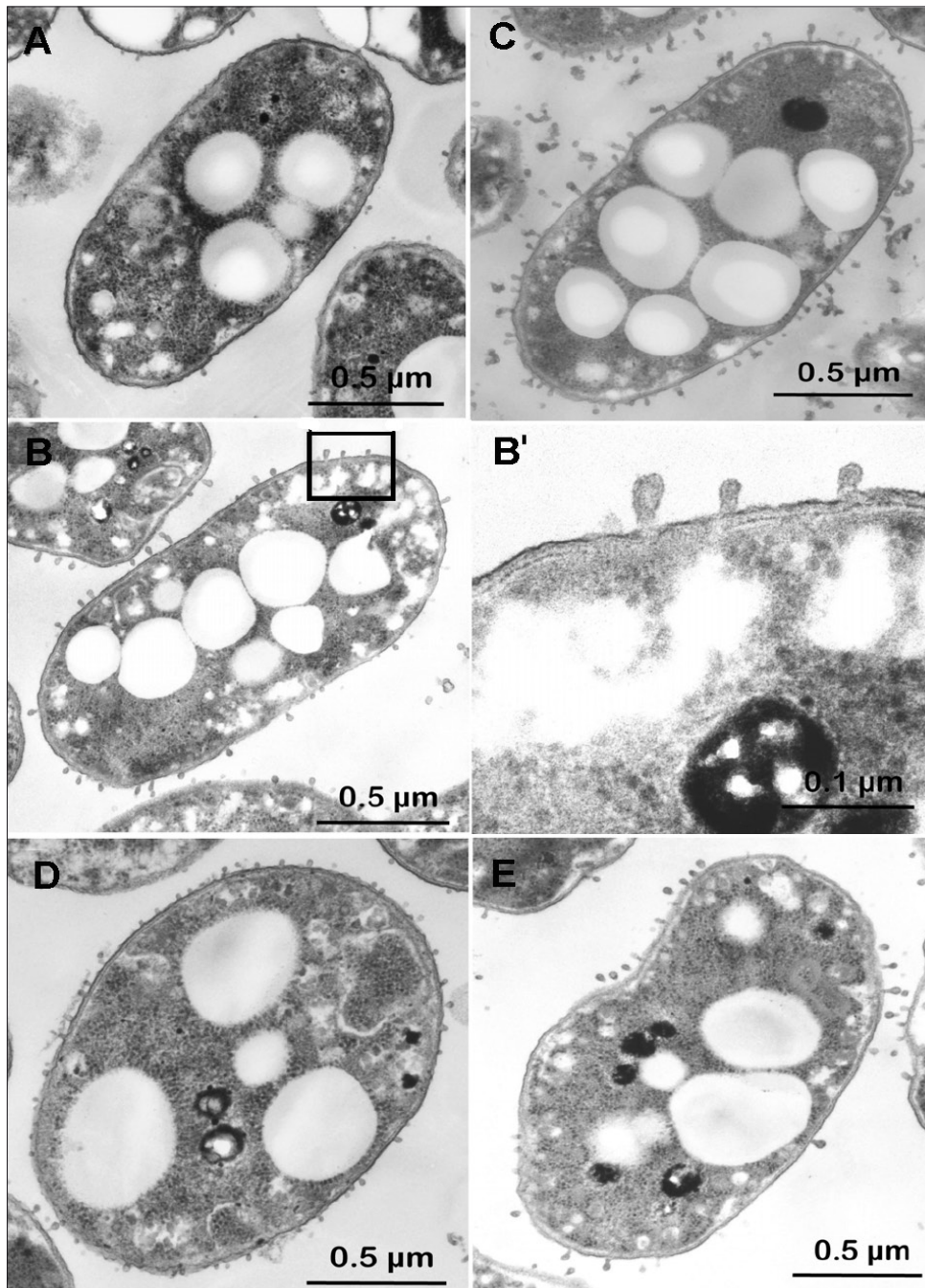
ing the disrupted *algD* gene was negligible. Mutant cells formed nonmucoid colonies on nitrogen-free MB medium solidified with agar, in contrast to the mucoid colonies of wild-type cells (Fig. 2). However, the growth of wild-type vs. mutant cells did not differ (Fig. 3A), indicating that *algD* disruption had no influence on either bacterial growth or nitrogen fixation.

Both the wild-type and the mutant cells were cultivated in MB medium under atmospheric air conditions, N79/O21, N75/O25, N50/O50, N100, or O100. In medium exposed for 5 min to a gas mixture of increasing oxygen content, the dissolved oxygen concentration increased accordingly (Fig. 1B). The growth of wild-type cells under atmospheric air conditions was essentially the same as that of cells exposed for 5 min at 8 h intervals to N79/O21. As expected, wild-type and mutant cells exposed to N100 or O100 did not grow in nitrogen-free medium since *A. vinelandii* is an aerobic, nitrogen-fixing bacterium. Increased oxygen levels (21 %, 25 %, and 50 %) were accompanied by a decrease in growth of both the wild-type and the mutant cells (Fig. 3A). Similar growth patterns were observed in the plate cultures exposed to the gas mixtures and the mucoid phenotype of each strain did not change in re-

sponse to the different gas ratios tested (Fig. 2). Wild-type and mutant cells were also cultivated aerobically up to the exponential growth phase in the MB medium and subsequently grown under atmospheric air conditions, N25/O50, and N25/O75 (Fig. 3B). The poor growth of either culture under N25/O75 conditions was consistent with a lack of protection from oxygen damage rather than an insufficiency of nitrogen gas. Therefore, in general, there was little difference in growth between wild-type and mutant cells exposed to the different gas mixtures.

For stationary phase cultures of both strains, growth under high oxygen conditions (25 %) yielded amounts of alginate larger than those obtained under atmospheric air conditions (atmospheric air: 0.215 mg/ml; N50/O25: 0.646 mg/ml). However, the alginate content was higher under N50/O25 than under N50/O50 (0.381 mg/ml). The higher alginate content in the culture broth under N50/O25 conditions in comparison with that of N50/O50 was dependent on the growth of the bacterial cultures.

**Response to nitrogen level.** The growth of bacterial cells exposed to N75/O25 or N50/O25 was comparable to that of cultures under atmospheric air conditions (Fig. 3A,C).



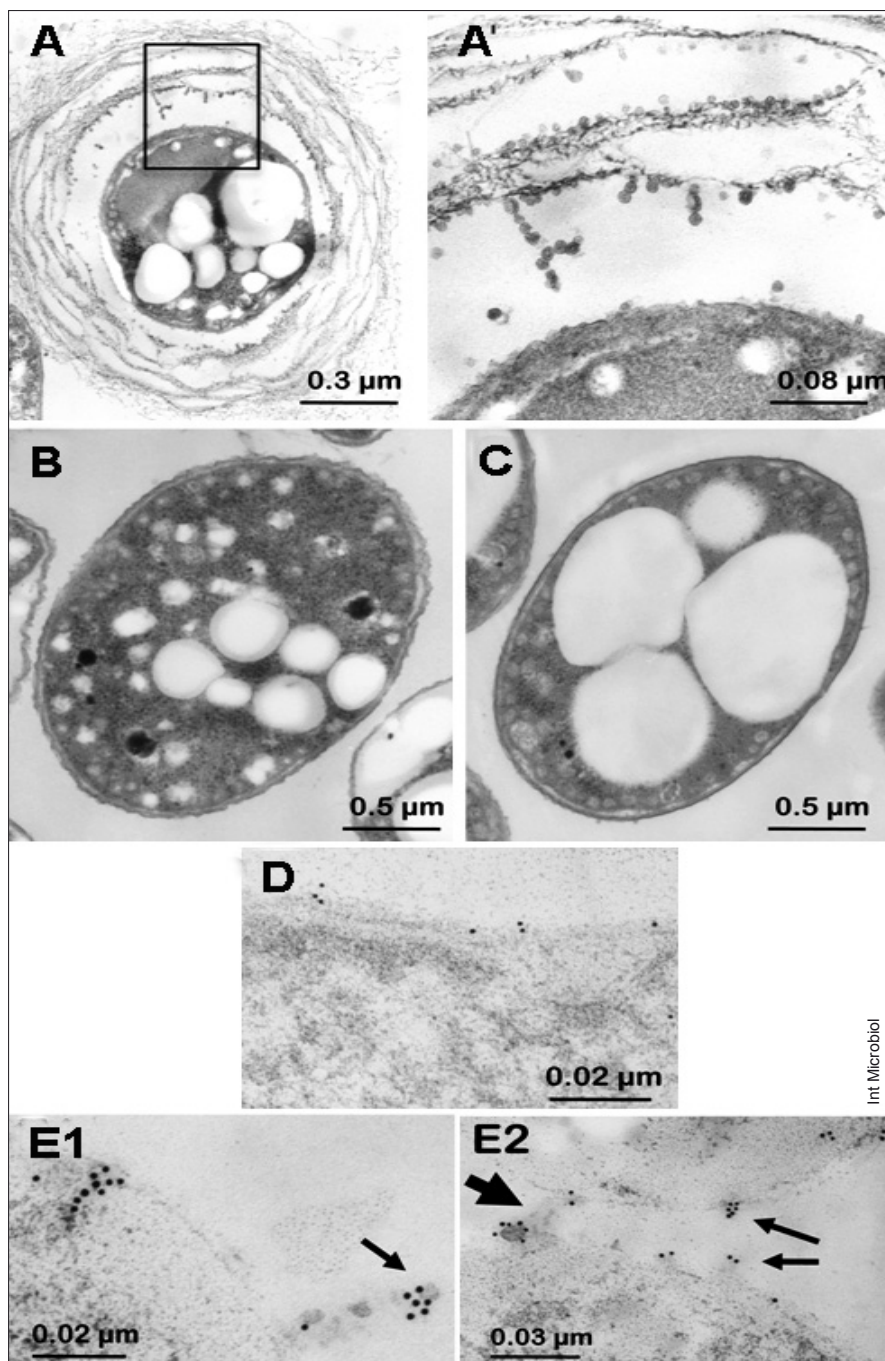
**Fig. 4.** Bleb formation on the cell surface of *Azotobacter vinelandii*. Wild-type cells were grown to stationary phase in the presence of various gas mixtures. Thin sections were prepared from fixed cells and observed by electron microscopy. (A) Atmospheric air. (B) N5/O25. (B') Magnification of the boxed region in (B). (C) N5/O25. (D) N5/O50. (E) N25/O50.

Note that *A. vinelandii* cells were able to grow even when exposed to N5/O25, a slight delay was observed in the exponential growth phase in comparison with bacterial cells exposed to N75/O25 and N50/O25.

Alginate production seemed to be dependent on the nitrogen level, with lower nitrogen ratios associated with increased alginate synthesis (N75/O25: 0.220 mg/ml; N50/O25: 0.646 mg/ml; N5/O25: 0.793 mg/ml). Under atmo-

spheric air conditions, *A. vinelandii* formed rod-shaped cells and produced polyhydroxybutyrate (PHB), as detected by electron microscopy of thin sections (Fig. 4A). In cells incubated under low nitrogen (N50/O25 and N5/O25) (Fig. 4B,C) or high oxygen (N25/O50) (Fig. 4D,E) conditions, a large number of blebs formed on the cell surface whereas fewer blebs were observed on bacterial cells grown under atmospheric air (Fig. 4A).





**Fig. 5.** Bleb-dependent alginate export in *Azotobacter vinelandii*. Wild-type (A, D, E1, and E2) and alginate-deficient mutant (B and C) cells were grown to stationary phase in the presence of various gas mixtures. Thin sections were prepared from fixed cells and observed by electron microscopy. The sections were further stained with anti-alginate antibodies for immunogold electron microscopy (D, E1, and E2). (A) Wild-type cyst formation (N25/O50). (A') Magnification of the boxed region in (A). (B) Mutant cell (atmospheric air). (C) Mutant cell (N50/O50). (D) Wild-type cell (atmospheric air). (E1 and E2) Wild-type cell (N5/O25). Thin arrows indicate alginate contained in blebs; the thick arrow points to bleb fusion at the interface of neighboring cells.

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In addition, some cells exposed to N25/O50 accumulated higher amounts of PHB in the cytoplasm and produced extracellular multilayered biofilms, indicating the conversion of vegetative cells to the cyst form (Fig. 5A).

**A novel alginate secretion system involving the formation of cell-surface blebs.** *A. vinelandii* cells incubated under nitrogen concentrations lower than the atmospheric concentration formed blebs (Fig.

4B–E) and the alginate concentration in the culture broth increased. In cells exposed to N25/O50, bleb formation was followed by the formation of an extracellular multilayered biofilm (Fig. 5A'), suggesting that the blebs contained alginate polysaccharides. The mutant cells did not form blebs when incubated in various gas mixtures, including atmospheric air and N50/O50 (Fig. 5B, C). Immunoelectron microscopy was carried out to directly examine the pres-



ence of alginate in the blebs of wild-type cells exposed to N5/O25. The accumulation of gold particles in the blebs (Fig. 5E1 and 5E2, arrows) confirmed that they contained alginate. In wild-type cells grown under atmospheric air conditions, the polysaccharide was distributed on the cell surface (Fig. 5D).

## Discussion

In this study, we investigated the responses of *A. vinelandii* cells to incubation with gas mixtures differing in their percentages of nitrogen and oxygen. Specifically, cell growth, the concentration of alginate in the culture broth, and cell structure at the stationary growth phase in response to several different gas conditions were examined in order to better understand the bacterial response to nitrogen levels higher or lower than atmospheric conditions.

Alginate biofilms are thought to protect bacterial cells from oxygen damage at limited phosphate levels [24]. However, even at high levels of oxygen (N50/O50 and N25/O50) and normal phosphate levels, both the wild-type and the alginate-deficient mutant cells were able to grow quite well, suggesting that alginate does not contribute to the protection of nitrogenases from oxygen damage.

*Azotobacter vinelandii* cells exposed to high oxygen levels (50 %) were round rather than rod-shaped (Fig. 4). In the presence of low nitrogen/high oxygen, vegetative cells formed cysts by synthesizing extracellular multilayered biofilms. Together with an increase in respiration at the exponential growth phase, encystment at the stationary growth phase is an important mechanism by which bacteria protect themselves from excess oxygen influx at high oxygen concentrations.

In *A. vinelandii* cells, several stress factors, such as desiccation [10], cause a shift from vegetative cells to alginate-coated cysts [25]. Similarly, changes in the gas composition from atmospheric conditions, e.g., to low nitrogen/high oxygen, also trigger cyst formation in *A. vinelandii*. In our study, alginate polysaccharide accumulated in the blebs formed on the cell surface during encystment. Release of the blebs from the cell surface was accompanied by the formation of multilayered biofilms that surrounded the bacterial cells. This system of direct alginate export in *A. vinelandii* through cell-surface alterations can be regarded as the counterpart of the one allowing alginate uptake in *Sphingomonas* sp. strain A1, which we characterized in a previous study [1]. This gram-negative sphingo-

monad accumulates external alginate in mouth-like pits that form on the cell surface, allowing the transport of polysaccharide across the outer and inner membranes into the cytoplasm by a periplasmic binding-protein-dependent ATP-binding cassette (ABC) transporter [17]. Subsequently, alginate is degraded into monosaccharides by four alginate lyases [9]. These unusual cell-surface alterations in these two species of bacteria may be related to the unique rheological properties (e.g., gel-sol transition and/or chelator) of alginate.

In gram-negative bacteria, macromolecules such as DNA and proteins are secreted through the formation of cell-surface blebs or membrane vesicles [16], but to our knowledge, a similar bleb-dependent system that mediates polysaccharide secretion has not been described. Following the initial report in the early 1970s of bleb formation during encystment [10], there have been no further investigations into this process, and the components of the blebs have not been determined. This may have been due to the inability to obtain stable blebs in cultured bacteria. In our study, the regulation of gas levels under the culture conditions described here resulted in stable blebs and thus insights into their contents.

*Haemophilus influenza* shows a natural competence for DNA and transfers DNA to recipient cells through its release from cell-surface blebs [12]. As an example of protein secretion through bleb formation, the cell-surface blebs of *P. aeruginosa* contain autolysin, the release of which results in the killing of other bacterial cells [15]. Analogous to the “transformasomes”, i.e., the DNA-containing bleb-like structures on the cell surface of *H. influenzae* [12], we have named the novel alginate export system of *A. vinelandii* as a “transalgisome.”

Gram-negative bacteria often release outer membrane blebs or vesicles 0.5–1 nm in diameter into the culture medium. These blebs typically contain enzymes or signaling molecules that are transferred to other bacterial cells when the vesicles fuse to the outer membrane of the recipient cells, providing a mechanism for prokaryotic cell–cell communication [16]. This has been observed in *Bacillus subtilis*, which transfers cytoplasmic molecules to neighboring *B. subtilis* but also to *Staphylococcus aureus*, and *E. coli* cells, through intercellular nanotubes formed on the cell surface [5]. A similar process may occur in *A. vinelandii* cells, in which blebs were found to mutually fuse at the interface of neighboring cells (Fig. 5E2, thick arrow). Note that bleb fusion provides a means of cell–cell communication, e.g., in the conversion of a population of vegetative

cells to cysts.

In conclusion, the ability of *A. vinelandii* cells grown at normal phosphate concentrations to fix nitrogen indicated that alginate did not contribute to the protection of oxygen-sensitive nitrogenases from oxygen damage. Gas stress, such as induced by low nitrogen/high oxygen levels, triggered bleb formation on the surface of these bacterial cells. During encystment, alginate contained in membrane vesicles was secreted via cell-surface blebs, resulting in aggregation of the polysaccharide, which constituted the exine of cysts. To our knowledge, this is the first report of a bacterial bleb-dependent polysaccharide export system.

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**Competing interests:** None declared.

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