The Na⁺-translocating NADH : ubiquinone oxidoreductase of *Azotobacter vinelandii* negatively regulates alginate synthesis

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Azotobacter vinelandii is a nitrogen-fixing soil bacterium that produces the exopolysaccharide alginate. In this report we describe the isolation and characterization of *A. vinelandii* strain GG4, which carries an *nqrE*::Tn5 mutation resulting in alginate overproduction. The *nqrE* gene encodes a subunit of the Na⁺-translocating NADH : ubiquinone oxidoreductase (Na⁺-NQR). As expected, Na⁺-NQR activity was abolished in mutant GG4. When this strain was complemented with the *nqrEF* genes this activity was restored and alginate production was reduced to wild-type levels. Na⁺-NQR may be the main sodium pump of *A. vinelandii* under the conditions tested (~2 mM Na⁺) since no Na⁺/H⁺-antiporter activity was detected. Collectively our results indicate that in *A. vinelandii* the lack of Na⁺-NQR activity caused the absence of a transmembrane Na⁺ gradient and an increase in alginate production.

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INTRODUCTION

Alginates are a family of unbranched, non-repeating copolymers composed of variable amounts of (1,4)- β -D-mannuronic acid and its epimer, α -L-guluronic acid. These biopolymers have various technological applications, e.g. as a stabilizing, thickening and gelling agent in food production or for immobilizing cells in the pharmaceutical and biotechnology industries (Rehm & Valla, 1997; Remminghorst & Rehm, 2006).

Azotobacter vinelandii is a nitrogen-fixing soil bacterium that produces the intracellular polymer polyhydroxybutyrate (PHB) and excretes copious amount of alginate into the medium during vegetative growth. Alginate is also produced when this bacterium undergoes differentiation into metabolically dormant, desiccation-resistant cysts (Sadoff, 1975). The cysts are surrounded by a rigid coat consisting of two layers mainly composed of alginate, and hence a mutant unable to produce alginate fails to encyst (Campos *et al.*, 1996; Mejía-Ruiz *et al.*, 1997a).

In *A. vinelandii* all genes involved in the biosynthesis of alginate, except *algC*, are clustered in the chromosome and are headed by *algD*, encoding a GDP-mannose dehydrogenase, an enzyme catalysing the committed step in the biosynthesis of

this polymer (Campos *et al.*, 1996). Transcription of *algD* and *algC* is positively controlled by the extracytoplasmic function sigma factor AlgU. *algD* expression is also controlled by the two-component system GacS/GacA, which regulates secondary metabolism in many Gram-negative bacteria (Castañeda *et al.*, 2000, 2001; Gaona *et al.*, 2004).

In *A. vinelandii*, as in *Pseudomonas aeruginosa*, AlgU is essential for alginate production and is encoded in the *algUmucABCD* operon (Martínez-Salazar *et al.*, 1996; Schurr *et al.*, 1996); MucA is an inner-membrane protein that acts as an anti-AlgU sigma factor by directly sequestering AlgU and inhibiting its activity. An *algU* mutant of *A. vinelandii* was unable to synthesize alginate. In contrast, a *mucA* mutation increased *algD* and *algC* transcription and consequently alginate production (Gaona *et al.*, 2004; Núñez *et al.*, 2000a).

Other mutations resulting in an increase of alginate production in *A. vinelandii* have been reported (Núñez *et al.*, 2000b; Segura *et al.*, 2003); a Tn5 insertion within *ampD*, encoding a cytosolic N-acetyl-anhydromuramyl-L-alanine amidase involved in the recycling of peptidoglycan fragments, increased *algD* transcription and alginate production. In addition a mutation within the structural gene *phbB*, which impaired PHB production, increased alginate yields, presumably as a consequence of higher carbon source availability for the synthesis of alginate (Segura *et al.*, 2003).

Abbreviations: Δ pH, transmembrane difference in pH; Na⁺-NQR, Na⁺-translocating NADH : ubiquinone oxidoreductase; NDH, NADH : quinone oxidoreductase; PHB, polyhydroxybutyrate.

Na^+ -translocating NADH:ubiquinone oxidoreductases (Na^+ -NQR) are redox-driven sodium pumps composed of six subunits, NqrA–F, which are encoded in the *nqr* operon (Hayashi *et al.*, 1995; Nakayama *et al.*, 1998; Rich *et al.*, 1995). This enzyme couples the exergonic oxidation of NADH with ubiquinone to the transport of Na^+ from the cytoplasmic to the periplasmic space (Skulachev, 1989). Thus, Na^+ -NQR diminishes the internal Na^+ concentration and contributes to the generation of an electrochemical Na^+ potential. This sodium transmembrane potential plays a significant role in the metabolism of various bacteria, in which it is involved in solute import, ATP synthesis and flagellar rotation (Skulachev, 1989). In *Vibrio cholerae* the sodium-motive force, which is maintained by Na^+ -NQR, strongly influences the production of virulence factors. Loss of Na^+ -NQR results in altered virulence gene regulation in *V. cholerae*, but the putative link between sodium membrane energetics and virulence remains unclear (Häse & Mekalanos, 1999). Although Na^+ -NQR-type enzymes have been investigated primarily in members of the genus *Vibrio* (Barquera *et al.*, 2002; Bogachev *et al.*, 2001, 2002; Duffy & Barquera, 2006; Häse & Barquera, 2001; Tokuda & Unemoto, 1982) it has recently been shown that homologous *nqr* operons are present in a variety of bacteria, including several pathogens such as *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *P. aeruginosa* and *Chlamydia trachomatis* (Dibrov *et al.*, 2004; Häse *et al.*, 2001).

The *nqr* operon is also found in *A. vinelandii*. In this report we describe the isolation of an *A. vinelandii* Tn5 mutant strain carrying an insertion within the *nqrE* gene, encoding a subunit of the Na^+ -NQR complex, that resulted in alginate overproduction.

METHODS

Microbiological procedures. Bacterial strains and plasmids are listed in Table 1. Media and growth conditions were as follows. *A. vinelandii* was grown at 30 °C in Burk's nitrogen-free salts supplemented with 2 % sucrose (BS) or in BS medium supplemented with 15 mM ammonium acetate (BSN) (Kennedy *et al.*, 1986). *Escherichia coli* DH5 α was grown on Luria–Bertani (LB) medium at 37 °C (Miller, 1972). Antibiotic concentrations used for *A. vinelandii* and *E. coli*, respectively, were as follows: spectinomycin, 50 and 100 $\mu\text{g ml}^{-1}$; gentamicin, 0.5 and 10 $\mu\text{g ml}^{-1}$; ampicillin, 100 $\mu\text{g ml}^{-1}$ (not used for *A. vinelandii*).

A. vinelandii transformation was carried out as previously described (Page & von Tigerstrom, 1978) with some modifications of the method. In brief, *A. vinelandii* was cultured twice on solid competence medium (CM; BS medium lacking Fe and Mo) followed by a subculture into 50 ml liquid CM. Cells were harvested and resuspended in 0.5 ml CM containing 16 mM MgSO_4 ; 100 μl of this suspension was transformed with 5 μg plasmid DNA. This mix was spread over CM plates and incubated at 30 °C for 24 h. Cells were then recovered and resuspended in 1 ml MgSO_4 . *A. vinelandii* transformants were isolated on BS plates containing the corresponding antibiotic.

Alginate production was determined as previously described (Mejía-Ruiz *et al.*, 1997b).

Transposon mutagenesis. Random transposon mutagenesis of *A. vinelandii* strain AEIV was carried out using the minitransposon mTn5SSgusA40 (Wilson *et al.*, 1995). Strain AEIV was cultured in 50 ml BS medium for 48 h and then cells were harvested and resuspended in 1 ml 10 mM MgSO_4 . *E. coli* strain S17-1 λ -pir carrying the mini Tn5SSgusA40 transposon (Wilson *et al.*, 1995) was grown for 24 h in 5 ml LB medium amended with spectinomycin. The cells were then washed and resuspended in 1 ml fresh LB medium. Aliquots (100 μl each) of the *E. coli* and *A. vinelandii* cell suspensions were mixed and spread over the surface of a BS plate containing 30 % (v/v) LB medium and 0.2 % glucose. The cells were incubated for 24 h at 30 °C to allow conjugation. The resultant *A. vinelandii* transconjugants were isolated on BS plates containing spectinomycin. Since BS medium lacks a fixed N source, *E. coli* cells were unable to grow.

Nucleic acid procedures. Genomic DNA was extracted with the MasterPure Complete DNA & RNA Purification kit (Epicentre Technologies). Plasmid DNA and PCR products were purified using the Roche High Pure plasmid isolation kit and the rapid PCR purification system (Marligen Biosciences), respectively. DNA cloning and other manipulations were carried out according to the methods outlined in Sambrook *et al.* (1989). Probes for Southern blot analysis were [α - ^{32}P]dCTP labelled with the Rediprime II kit from GE Healthcare. The *A. vinelandii* genome sequence is available via the Integrated Microbial Genome Sequence web page (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>) and this sequence was used for designing the oligonucleotides used for PCR amplifications. *Taq* DNA polymerase was used for all PCR amplifications.

Cloning the transposon insertion. Transposon mTn5SSgusA40 lacks sites for the *Pst*I endonuclease. Chromosomal DNA from *A. vinelandii* GG4 was restricted with this enzyme. The *Pst*I chromosomal fragment interrupted by the transposon was ligated into pBluescript KS $^+$ (Table 1) and was transformed into *E. coli* DH5 α . A spectinomycin-resistant transformant harbouring plasmid pGGCN4 was isolated (Table 1). This plasmid was used to determine the sequence of the interrupted locus in the GG4 mutant. DNA sequencing was performed as described, using oligonucleotides Tn5I (5'-GGC CAG ATC TGA TCA AGA G-3') and Tn5O (5'-GGC CGC ACT TGT GTA TAA G-3') (Núñez *et al.*, 2000b). Sequence analysis revealed that the Tn5 insertion in *A. vinelandii* GG4 lies within a 930 bp *Pst*I fragment (Fig. 1a).

Construction of mutant ATCN4. *A. vinelandii* ATCC 9046 was transformed with plasmid pGGCN4, carrying the *nqrE*::Tn5 insertion (Fig. 1a). To ensure that incorporation of the disrupted *nqrE* coding sequence into the chromosome occurred via a double recombinant event, pGGCN4 was linearized with *Pst*I prior to transformation. Transformants were selected on BS plates amended with spectinomycin. The replacement of the wild-type *nqrE* gene by the *nqrE*::Tn5 insertion was confirmed by Southern blotting (data not shown), and the resultant strain was named ATCN4.

Construction of plasmid pCN41. The *nqrE* and *nqrF* genes were amplified by PCR using oligonucleotides nqrEFF (5'-GCA AAC TGT TCG GCT TCA CC-3') and nqrEFR (5'-ATG GCG GGC TTC AGC AGT G-3') and an annealing temperature of 58 °C; genomic DNA from strain UW136 (Kennedy *et al.*, 1986) was used as the template. The *nqrEF* gene product was ligated into pMOSblue vector using the pMOSblue blunt-ended cloning kit (GE Healthcare), and the resultant plasmid was named pCN40. We have previously shown that genes located downstream of the Gm r cassette from plasmid pBSL141 (Alexeyev *et al.*, 1995) are transcribed from the promoter directing transcription of the gentamicin-resistance gene (Núñez *et al.*, 2000a). A *Bam*HI fragment containing this cassette was ligated into the

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source of reference
<i>A. vinelandii</i>		
AEIV	Wild-type, mucoid	S. Valla*
GG4	AEIV derivative carrying a <i>nqrE</i> ::Tn5 mutation	This work
CN14	GG4 derivative carrying pCN41 cointegrated in the chromosome	This work
ATCC 9046	Highly mucoid	ATCC
ATCN4	ATCC 9046 derivative carrying the <i>nqrE</i> ::Tn5 mutation	This work
NL17	ATCN4 derivative carrying pCN41 cointegrated in the chromosome	This work
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 hsdR17 recA1 endA gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
GR70N	F ⁻ <i>thi rpsL gal</i> ; Str ^R	Green <i>et al.</i> (1984)
Plasmids		
pBluescript KS ⁺	Used for subcloning DNA; Ap ^r	Stratagene
pMOSblue	Used for subcloning PCR products; Ap ^r	Amersham
pCAM140	mTn5Ssgusa40 (promoterless <i>gusA</i> for transcriptional fusions) in pUT/miniTn5Sm/Sp; Ap ^r	Wilson <i>et al.</i> (1995)
pCN40	PMOSblue derivative carrying a 2 kb fragment containing <i>nqrEF</i> genes	This work
pCN41	pCN40 derivative containing a Gm ^r cassette ligated into the polylinker	This work
pGGCN4	pBluescript KS ⁺ with the <i>PstI</i> fragment containing the <i>nqrE</i> ::Tn5 mutation	This work

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polylinker of pCN40 to direct transcription of the *nqrEF* genes. The resultant plasmid was named pCN41 (Fig. 1a).

Genetic complementation of mutants GG4 and ATCN4. Plasmid pCN41, carrying the *nqrEF* genes and unable to replicate in *A. vinelandii*, was used to complement *nqrE*::Tn5 mutants by its co-integration into the chromosome, as has been reported before (Núñez *et al.*, 1999; Segura & Espin, 1998). Competent *A. vinelandii* GG4 and ATCN4 cells were transformed with 5 μ g supercoiled pCN41 (Fig. 1b). Transformants in which pCN41 was co-integrated into the chromosome were isolated on BS plates containing gentamicin and spectinomycin, and co-integration was confirmed by Southern blotting (Fig. 1c). In all five candidates tested, co-integration of plasmid pCN41 had occurred downstream of the *nqrE*::Tn5 insertion. This event was expected, as the corresponding Tn5 downstream region in pCN41 is twice as long as the upstream one. One such candidate was chosen for further analysis and the resultant GG4 and ATCN4 derivative strains were named CN14 and NL17, respectively.

Preparation of sub-bacterial particles and determination of NADH dehydrogenase activity and Δ pH formation. *E. coli* and *A. vinelandii* cells were grown in LB and BSN media respectively, harvested by centrifugation (10 000 g, 10 min) and washed twice with medium 1 (250 mM KCl, 10 mM Tris/HCl and 5 mM MgSO₄, pH 8.0). The cell pellet was subsequently suspended in medium 2 (20 mM HEPES-Tris, 5 mM MgSO₄, 100 mM KCl, pH 8.0) and the suspension was passed through a French press (110 400 kPa). Undamaged cells and cell debris were removed by centrifugation at 22 500 g (10 min) and the supernatant was further centrifuged at 180 000 g for 60 min. The membrane pellet was suspended in medium 2 at 20–30 mg protein ml⁻¹. As shown previously (Bertsova & Bogachev, 2002) this procedure produces membrane vesicles with a mixed orientation with a ratio from 3:1 to 4:1 of ‘inside out’ to ‘right-side out’ particles. Since in all experiments we used a substrate (NADH) non-permeable through bacterial membranes, only ‘inside out’ oriented vesicles were active in our study. These vesicles have low H⁺ permeability and are suitable for investigation of Δ pH formation (Bertsova *et al.*, 1998).

The rates of NADH and dNADH oxidation by sub-bacterial particles were measured in medium 2 at 30 °C using a Hitachi-557 spectrophotometer as described previously (Bertsova & Bogachev, 2002).

Δ pH formation by sub-bacterial particles was monitored using acridine orange fluorescence quenching (MPF-4 fluorimeter: excitation, 492 nm; emission, 530 nm) (Bertsova *et al.*, 1998). The incubation mixture contained medium 2, 4 μ M acridine orange and sub-bacterial particles (40–60 μ g protein ml⁻¹). A 2 ml sample was incubated for 5 min and respiration was initiated by adding 2 mM NADH (Tris salt).

RESULTS

Isolation of the alginate-overproducing mutant GG4

A. vinelandii strain AEIV produces low levels of alginate, and therefore shows a slightly mucoid phenotype on BS plates (Campos *et al.*, 1996). Random Tn5 mutagenesis of this strain was conducted as described in Methods and a total of 4200 isolates (Sp^r) were obtained. This bank of mutants was screened for derivatives showing a highly mucoid colony morphology. Strain GG4, which had a stable alginate-overproducing phenotype, was chosen for further analysis. Its specific alginate production was 60-fold higher than that of the parental strain AEIV after 48 h of growth on solid BS medium [3200 vs 52 μ g alginate (mg protein)⁻¹; Table 2].

Na⁺-NQR negatively regulates alginate synthesis

The *PstI* fragment carrying the site of transposition in mutant GG4 was subcloned and the sequence of the

252

Strain	Alginate production [$\mu\text{g (mg protein)}^{-1}$]*
AEIV	52 ± 8
GG4 (<i>nqrE</i> ::Tn5)	3200 ± 203
CN14 (<i>nqrEF</i> ⁺)	64 ± 12
ATCC 9046	800 ± 84
ATCN4 (<i>nqrE</i> ::Tn5)	3400 ± 422
NL17 (<i>nqrEF</i> ⁺)	1406 ± 58

(Fig. 1a). As shown in Table 2, the resultant strain CN14 exhibited low alginate levels similar to those observed for wild-type strain AEIV.

ATCC 9046 is a highly mucoid strain of *A. vinelandii*, producing approximately 10 times more alginate than strain AEIV (Table 2). Strain ATCC 9046 was transformed with plasmid pGGCN4, carrying the *nqrE*::Tn5 insertion, and mutant ATCN4 was isolated as described in Methods. Elimination of the Na⁺-NQR complex in the background of strain ATCC 9046 increased alginate production to levels similar to those observed in mutant GG4 (Table 2). Furthermore, genetic complementation of mutant ATCN4 with the *nqrEF* genes diminished alginate production to a level slightly higher than that of the ATCC 9046 wild-type strain. These results indicate that the negative effect of the Na⁺-NQR complex on the biosynthesis of alginate is not strain specific.

A BLAST search of the draft sequence of the *A. vinelandii* genome (<http://img.jgi.doe.gov/cgi-bin/pub/main.cgi>) revealed that as well as the *nqr* genes, this bacterium contains genes for two additional respiratory-chain-linked NADH:quinone oxidoreductases (NDH): a gene encoding an NDH-2 type (GenBank accession number ZP_00415587), and 13 genes encoding the subunits of NDH-1 (GenBank accession numbers from ZP_00417514 to ZP_00417526). NDH-1- and NDH-2-type enzymes have been shown to function in *A. vinelandii* (Bertsova *et al.*, 2001), whereas the activity of the Na⁺-NQR-type enzyme had not been previously detected.

Microbiology 155

dNADH (Bertsova & Bogachev, 2004); however, NDH-1 enzyme activity is specifically inhibited by rolliniastatin (Degli Esposti *et al.*, 1994). NDH-2-type enzymes generally oxidize only NADH, but not dNADH (Bertsova & Bogachev, 2004). To determine whether the Na⁺-NQR was functional in *A. vinelandii*, either NADH- or dNADH-oxidase activities of strains AEIV and GG4 were studied. Sub-bacterial particles from *A. vinelandii* AEIV oxidized either NADH or dNADH with rates of 2.1 and 0.9 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹, respectively. As can be seen from Fig. 2, dNADH oxidase activity of strain AEIV was only partially sensitive to rolliniastatin. Sub-bacterial particles from *A. vinelandii* GG4 (*nqrE*::Tn5) also oxidized NADH as well as dNADH [with respective rates of 2.4 and 0.5 $\mu\text{mol min}^{-1}$ (mg protein)⁻¹]. However, in contrast to the wild-type strain AEIV or the complemented strain CN14, the dNADH oxidase activity of *A. vinelandii* GG4 was completely inhibited by rolliniastatin (Fig. 2), and by low concentrations of piericidin A (data not shown). It has also been shown that rolliniastatin-resistant NADH oxidase activity is specifically stimulated by Na⁺ (Fadeeva *et al.*, 2008). These data imply that there are two different enzymes capable of dNADH oxidation (NDH-1 and Na⁺-NQR) in wild-type *A. vinelandii*, while the Na⁺-NQR enzyme is absent in the GG4 mutant. Taken together these results demonstrate that a Na⁺-NQR-type enzyme, encoded by the *nqr* gene cluster, operates in the respiratory chain of *A. vinelandii*.

Na⁺/H⁺-antiporter activity is not detectable in *A. vinelandii* at low Na⁺ concentrations

In the majority of micro-organisms, the transmembrane Na⁺ gradient is formed by secondary processes such as Na⁺/H⁺-antiport (Padan *et al.*, 2001). Therefore, we

Fig. 2. Effect of rolliniastatin on the dNADH oxidase activities of sub-bacterial particles from *A. vinelandii* AEIV (●), GG4 (■) and CN14 (▲). Activities were measured in medium containing 20 mM HEPES-Tris, 5 mM MgSO₄, 100 mM KCl and 20 mM NaCl, pH 8.0. The activity without the inhibitor for sub-bacterial particles from strains AEIV, GG4 and CN14 was 0.9, 0.5 and 0.8 $\mu\text{mol dNADH oxidized min}^{-1}$ (mg protein)⁻¹, respectively. Error bars represent standard deviation for triplicate experiments.

measured the Na⁺/H⁺-antiporter activity in *A. vinelandii* by comparing the properties of *A. vinelandii* sub-bacterial particles to those of *E. coli*, which expresses Na⁺/H⁺-antiporters. As can be seen in Fig. 3(a), NADH oxidation by sub-bacterial particles of *E. coli* in sodium-free medium resulted in ΔpH formation. Subsequent NaCl addition led to a transient decrease in ΔpH due to Na⁺/H⁺-antiporter activity (Dibrov *et al.*, 2005). In contrast, addition of NaCl to respiring sub-bacterial particles from wild-type *A. vinelandii* AEIV did not change the ΔpH formed (Fig. 3b). The same results were also obtained using sub-bacterial particles from the *A. vinelandii* GG4 mutant (Fig. 3c). These data indicate that, under the condition tested (~2 mM Na⁺), Na⁺/H⁺-antiporter activity is very low or absent in *A. vinelandii* and suggest that the Na⁺-NQR complex is the main Na⁺ pump necessary to maintain a transmembrane Na⁺ gradient on the cytoplasmic membrane of this bacterium. This result implies that the absence of a transmembrane Na⁺ gradient in the *nqrE*::Tn5 mutant might increase alginate production.

Effect of osmolarity on alginate synthesis

To assess the effect of exogenous Na⁺ upon alginate production *A. vinelandii* wild-type strains AEIV and ATCC 9046, and their *nqrE*::Tn5 derivative mutants, were grown on BS plates amended with increasing concentrations of NaCl. Alginate production was measured on cultures grown for 72 h. As shown in Table 3, exogenous Na⁺ had a negative effect on alginate biosynthesis; a complete impairment of alginate synthesis was achieved at 150 mM NaCl for *A. vinelandii* wild-type strains AEIV and ATCC 9046, which exhibited alginate production values of 2.4 and 2.9 $\mu\text{g (mg protein)}^{-1}$, respectively. These values are similar to those obtained for alginate-minus mutants of *A. vinelandii* (Mejía-Ruiz *et al.*, 1997b). Alginate production in mutants GG4 and ATCN4 was also inhibited in the presence of 50 mM NaCl; however, it was not totally impaired at 150 mM NaCl. The GG4 complemented strain CN14 regained the total inhibition of alginate synthesis in the presence of 150 mM NaCl.

In order to test whether the negative effect of exogenous NaCl on alginate production was due to the presence of Na⁺ or was a reflection of changes in medium osmolarity we conducted the same experiment using KCl. Alginate production was inhibited in the presence of this salt in a manner similar to that observed in the presence of NaCl (data not shown). This result indicates that alginate production is negatively affected by an increase in medium osmolarity rather than by the presence of high concentrations of exogenous Na⁺.

DISCUSSION

Prior to this work, we reported that either a total blockade in PHB synthesis, a defect in the cell wall or mutations disrupting the genes *mucABCD* resulted in high levels of

Fig. 3. Na^+/H^+ -antiporter activity in sub-bacterial particles of *E. coli* GR70N (a), *A. vinelandii* AEIV (b) and *A. vinelandii* GG4 (c). ΔpH was monitored with acridine orange in a medium containing 20 mM HEPES-Tris (pH 8.0), 5 mM MgSO_4 , 100 mM KCl, 4 μM acridine orange and sub-bacterial particles (40–60 μg protein ml^{-1}). At the onset of the experiment, Tris salt of NADH (2 mM) was added, and the fluorescence quenching (ΔF) was recorded. NaCl (10 mM) was then added, and the new steady state of fluorescence obtained (dequenching) was monitored. When indicated, the artificial Na^+/H^+ -antiporter monensin (5 μM) was added.

alginate production (Núñez *et al.*, 2000a, b; Segura *et al.*, 2003). The current work demonstrates that the absence of Na^+ -NQR enzyme activity also results in increased alginate production. In halophilic bacteria such as *Vibrio* spp., Na^+ -NQR activity has an important role in both the environmental and pathogenic phases of their life cycle due to their ability to use sodium transmembrane potential for bioenergetic processes (Häse & Barquera, 2001; Yorimitsu & Homma, 2001). *A. vinelandii* is not halophilic; indeed the BS growth medium contains only 2.6 mM Na^+ and growth is abrogated at concentrations higher than 200 mM NaCl (data not shown). In a recent study, characterization of the catalytic properties of the *A. vinelandii* Na^+ -NQR enzyme indicated that the Na^+ -NQR enzyme of *A. vinelandii* possesses the highest affinity for Na^+ so far described, with an apparent K_m of 0.1 mM, an affinity 27 and 6 times higher than those from *Vibrio harveyi* and *Klebsiella pneumoniae*, respectively (Fadeeva *et al.*, 2008). This result is consistent with the low concentrations of Na^+ present in the natural habitat of *A. vinelandii*. In the present work we found that in *A. vinelandii* the activity of

Na^+/H^+ -antiporters is either very low or absent in media containing low Na^+ concentrations (~ 2 mM). Analysis of the genome sequence of *A. vinelandii* revealed the existence of four genes encoding putative Na^+/H^+ -antiporters (data not shown). This indicates that, under our tested condition, these genes are not expressed and that the Na^+ -NQR complex is likely to be the main sodium pump. Thus in mutant GG4, which lacks Na^+ -NQR, the absence of a transmembrane sodium gradient might be a signal that triggers alginate production (see below). Another possibility is that the lack of the Na^+ -NQR in mutant GG4 decreases the overall NADH oxidase activity of this bacterium. However, this is unlikely, as the Na^+ -NQR activity in *A. vinelandii* is very low when compared to that of NDH-2, the main NADH-consuming enzyme of this bacterium (Bertsova *et al.*, 1998). In addition, mutant GG4 is capable of diazotrophic growth at high oxygen concentrations, indicating that this mutant achieves the high respiration rate required for the respiratory protection of the nitrogenase complex (Bertsova *et al.*, 2001).

Table 3. Alginate production by *A. vinelandii* strains in the presence of exogenous NaCl

Added NaCl (mM)	Alginate production [μg (mg protein) $^{-1}$]*				
	AEIV	GG4 (<i>nqrE</i> ::Tn5)	CN14 (<i>nqrEF</i> $^{+}$)	ATCC 9046	ATCN4 (<i>nqrE</i> ::Tn5)
0	108 $\pm$ 7	20200 $\pm$ 400	66 $\pm$ 7	1125 $\pm$ 320	12459 $\pm$ 276
50	37 $\pm$ 3	6270 $\pm$ 540	45 $\pm$ 2	260 $\pm$ 80	6216 $\pm$ 120
150	2.4 $\pm$ 0.3	1350 $\pm$ 280	3.4 $\pm$ 1	2.9 $\pm$ 0.6	127 $\pm$ 35

*Alginate was determined in cells grown for 72 h on solid BS medium (initial Na^+ concentration 2.6 mM) in the presence of the indicated concentrations of NaCl. Data shown are the means $\pm$ SD from three replicate cultures.

The regulation of alginate synthesis by the Na⁺-NQR complex could be similar to the regulation of virulence factors in *V. cholerae*, which are positively controlled by ToxT. TcpP and TcpH are inner-membrane proteins that positively control transcription of *toxT*. Activation of *toxT* by TcpP/H is reduced at high NaCl levels and is increased in the absence of Na⁺-NQR complex activity (Häse & Mekalanos, 1999). As the Na⁺-NQR complex pumps out Na⁺, it was proposed that TcpP/H might sense elevated extracellular Na⁺ concentrations or some other associated signal to maintain low *toxT* transcription levels. Alternatively TcpP/H might be sensing the level of sodium gradient rather than high Na⁺ concentrations per se (Häse & Mekalanos, 1999). In this report we have shown that in *A. vinelandii* alginate production is inhibited by elevated extracellular Na⁺ concentrations; however, our results indicate that this is an effect associated with osmolarity. Further experiments will be necessary to understand the mechanism by which the Na⁺-NQR complex negatively controls alginate production in *A. vinelandii* and whether it involves the participation of elements similar to TcpP and TcpH of *V. cholerae* or transcriptional regulators, like the sigma factor AlgU and/or the global response regulator GacA, which are essential for alginate production (Castañeda *et al.*, 2000; Martínez-Salazar *et al.*, 1996). On the other hand, we cannot rule out the possibility that the absence of Na⁺-NQR activity leads to a more efficient secretion of alginate, by favouring the activity of the multi-enzyme complex involved in alginate polymerization, modification and export (Remminghorst & Rehm, 2006). Collectively our results indicate that in *A. vinelandii* the absence of Na⁺-NQR activity leads to the inability to maintain a transmembrane Na⁺ gradient and to an increase in alginate production.

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