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

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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 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\textsuperscript{+}-translocating NADH : ubiquinone oxidoreductase (Na\textsuperscript{+}-NQR). As expected, Na\textsuperscript{+}-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\textsuperscript{+}-NQR may be the main sodium pump of *A. vinelandii* under the conditions tested (~2 mM Na\textsuperscript{+}) since no Na\textsuperscript{+}/H\textsuperscript{+}-antiporter activity was detected. Collectively our results indicate that in *A. vinelandii* the lack of Na\textsuperscript{+}-NQR activity caused the absence of a transmembrane Na\textsuperscript{+} gradient and an increase in alginate production.

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).
Na\textsuperscript{+}-translocating NADH:ubiquinone oxidoreductases (Na\textsuperscript{+}-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\textsuperscript{+} from the cytoplasmic to the periplasmic space (Skulachev, 1989). Thus, Na\textsuperscript{+}-NQR diminishes the internal Na\textsuperscript{+} concentration and contributes to the generation of an electrochemical Na\textsuperscript{+} 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\textsuperscript{+}-NQR, strongly influences the production of virulence factors. Loss of Na\textsuperscript{+}-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\textsuperscript{+}-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\textsuperscript{+}-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., 1989). *Escherichia coli* DH5α 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 μg ml\textsuperscript{-1}; gentamicin, 0.5 and 10 μg ml\textsuperscript{-1}; ampicillin, 100 μg ml\textsuperscript{-1} (not used for *A. vinelandii*).

*A. vinelandii* transformation was carried out as previously described (Page & von Tigerstrom, 1978) with some modifications to 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\textsubscript{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\textsubscript{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 miritransposon mTnSSSgusA40 (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\textsubscript{4}. *E. coli* strain S17-1 λ-pir carrying the mini TnSSSgusA40 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-\textsuperscript{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 mTnSSSgusA40 lacks sites for the PstI endonuclease. Chromosomal DNA from *A. vinelandii* GG4 was restricted with this enzyme. The PstI chromosomal fragment interrupted by the transposon was ligated into pBluescript KS\textsuperscript{+} (Table 1) and was transformed into *E. coli* DH5α. 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 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 PstI 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 PstI 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 *nqrEF* (5’-GGC CAG ATC TGA AGA G-3’) and *nqrEF* (5’-ATG GGC 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 Gmr’ cassette from plasmid pBlSL141 (Alexeyev et al., 1995) are transcribed from the promoter directing transcription of the gentamicin-resistance gene (Núñez et al., 2000a). A BamHI fragment containing this cassette was ligated into the
**Table 1. Bacterial strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. vinelandii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEIV</td>
<td>Wild-type, mucoid</td>
<td>S. Valla*</td>
</tr>
<tr>
<td>GG4</td>
<td>AEIV derivative carrying a nqrE::Tn5 mutation</td>
<td>This work</td>
</tr>
<tr>
<td>CN14</td>
<td>GG4 derivative carrying pCN41 cointegrated in the chromosome</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 9046</td>
<td>Highly mucoid</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCN4</td>
<td>ATCC 9046 derivative carrying the nqrE::Tn5 mutation</td>
<td>This work</td>
</tr>
<tr>
<td>NL17</td>
<td>ATCN4 derivative carrying pCN41 cointegrated in the chromosome</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 hsdR17 recA1 endA gyrA96 thi-1 relA1</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>GR70N</td>
<td>F’ thi rpsL gal; StrR</td>
<td>Green et al. (1984)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript KS+</td>
<td>Used for subcloning DNA; Ap’</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMOSblue</td>
<td>Used for subcloning PCR products; Ap’</td>
<td>Amersham</td>
</tr>
<tr>
<td>pCAM140</td>
<td>mTn5SSgusA40 (promoterless gusA for transcriptional fusions) in pUT/miniTnSm/Sp; Ap’</td>
<td>Wilson et al. (1995)</td>
</tr>
<tr>
<td>pCN40</td>
<td>PMOSblue derivative carrying a 2 kb fragment containing nqrEF genes</td>
<td>This work</td>
</tr>
<tr>
<td>pCN41</td>
<td>pCN40 derivative containing a Gm’ cassette ligated into the polylinker</td>
<td>This work</td>
</tr>
<tr>
<td>pGGCN4</td>
<td>pBluescript KS+ with the PstI fragment containing the nqrE::Tn5 mutation</td>
<td>This work</td>
</tr>
</tbody>
</table>

*S. Valla, Norwegian University of Science and Technology, N-7491 Trondheim.

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).

ΔpH 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, 2 μM acridine orange and sub-bacterial particles (40–60 μg 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') 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 μg 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 polylinker of pCN40 to direct transcription of the nqrEF genes. The resultant plasmid was named pCN41 (Fig. 1a).

**Preparation of sub-bacterial particles and determination of NADH dehydrogenase activity and ΔpH 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 ΔpH formation (Bertsova et al., 1998).
flanking Tn5 regions was determined, as described in Methods. The sequence obtained revealed that the Tn5 insertion in mutant GG4 is located within the nqrE gene, which forms part of the Na\(^+\)-NQR gene cluster in *A. vinelandii* (*nqrA–F*), encoding the Na\(^+\)-NQR complex (GenBank accession numbers from ZP_00416255 to ZP_00416260). The transposon disrupted codon 94 (nucleotide 281) of the predicted NqrE protein and was inserted in the orientation opposite to that of nqrE transcription. Genetic complementation of mutant GG4 was conducted by co-integration into the chromosome of wild-type copies of both *nqrE* and *nqrF* genes transcribed from the promoter of the Gmr cassette of plasmid pCN41.

### Table 2. Alginate production by *A. vinelandii* wild-type strains and *nqrE*::Tn5 derivative mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alginate production [µg (mg protein)(^{-1})]</th>
</tr>
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<tbody>
<tr>
<td>AEIV</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>GG4 (<em>nqrE</em>::Tn5)</td>
<td>3200 ± 203</td>
</tr>
<tr>
<td>CN14 (*nqrEF(^+))</td>
<td>64 ± 12</td>
</tr>
<tr>
<td>ATCC 9046</td>
<td>800 ± 84</td>
</tr>
<tr>
<td>ATCN4 (<em>nqrE</em>::Tn5)</td>
<td>3400 ± 422</td>
</tr>
<tr>
<td>NL17 (*nqrEF(^+))</td>
<td>1406 ± 58</td>
</tr>
</tbody>
</table>

*Alginate was determined in cells grown for 48 h on solid BS medium. Data shown are the means ± SD from three replicate cultures.

Fig. 1. (a) Physical map of the *nqrEF* locus in *A. vinelandii* and of plasmids constructed in this work. (b) Schematic representation of the co-integration of plasmid pCN41 into the chromosome of GG4 in order to complement this mutant. (c) Southern blot hybridization of total genomic DNA digested with *Pst*I, with *nqrEF* PCR product as the probe. Lanes: 1, AEIV; 2, GG4; 3, CN14. The hybridization pattern of strain CN14 is consistent with that of the predicted pCN41 co-integration event downstream of the *nqrE*::Tn5 insertion as depicted in (b). Arrows indicate the direction of transcription. Antibiotic-resistance cassettes and Tn5 are not shown to scale. P, *Pst*I.

**Table 2. Alginate production by *A. vinelandii* wild-type strains and *nqrE*::Tn5 derivative mutants**

**Alginate production in the background of strain ATCC 9046**

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.

**Na\(^+\)-NQR activity in *A. vinelandii***

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.

The different types of NDH have different substrate specificity with respect to pyridine dinucleotides as well as different sensitivities to inhibitors. The NDH-1- and NQR-type enzymes oxidize NADH as well as its analogue...
dNADH (Bertsova & Bogachev, 2004); however, NDH-1 enzyme activity is specifically inhibited by rolliniastatin (Degl’Esposito 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 \(\mu\)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 \(\mu\)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 sodium 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 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 \(\Delta p\)H formation. Subsequent NaCl addition led to a transient decrease in \(\Delta p\)H 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 \(\Delta p\)H 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 \(\mu\)g (mg protein\(^{-1}\)), respectively. These values are similar to those obtained for alginate-minus mutants of _A. vinelandii_ (Mejia-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...
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).

![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.](image1)

<table>
<thead>
<tr>
<th>Added NaCl (mM)</th>
<th>Alginate production [μg (mg protein(^{-1}))]*</th>
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<tbody>
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<td></td>
<td>AEIV</td>
</tr>
<tr>
<td>0</td>
<td>108 ± 7</td>
</tr>
<tr>
<td>50</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>150</td>
<td>2.4 ± 0.3</td>
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</table>

*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 ± 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 (Remmingerst & 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.

**ACKNOWLEDGEMENTS**

We are grateful to Dr Maddalena V. Coppi and Dr Christopher Wood for critical reading of the manuscript. We thank K. Wilson for kindly providing the miniTnSSSgusA40 transposon, and S. Moreno and Arturo Ocaíz for their technical support. We also thank E. López, P. Gaytán and J. Yañez for the synthesis of primers and DNA sequencing. This research was supported by projects PAPIIT, no. IN214605 and IN214208 from the UNAM. A. V. B. is grateful to the Russian Foundation for Basic Research for financial support (grant 07-04-00619).

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Edited by: M. F. Hynes