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Alginate production by *Azotobacter vinelandii* mutants altered in poly-β-hydroxybutyrate and alginate biosynthesis

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Mutant AT268 of *Azotobacter vinelandii* — showing diminished production of poly-β-hydroxybutyrate (PHB) due to a mutation in *phbR* (the gene coding for the transcriptional activator of the *phbBAC* biosynthetic operon); mutant CNT26, containing a mutation (*muc26*) that increases the transcription of gene *algD* (encoding GDP mannose dehydrogenase, the key enzyme in alginate biosynthesis); and mutant DM, carrying both *phbR* and *muc26* mutations — were characterised in terms of alginate production, broth viscosity, and molecular weight of the alginate. All the mutants evaluated produced 25% less alginate with respect to that produced by the wild type. Unexpectedly and with no apparent relation to the *phbR* and *muc26* mutations, mutant DM exhibited the highest molecular weight ever reported for a bacterial alginate (up to 4×10^6 Da), with a very low polydispersity index (1.3). Acetyl content in the alginate produced by this strain was low (1.4–2.6%). These characteristics make this mutant a very valuable source for producing alginates with improved properties.
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Introduction

Alginates are polysaccharides of β-D-mannuronic acid and its C-5 epimer, α-L-guluronic acid, arranged in irregular blocks along the linear chain. The main applications of alginates are as thickeners, stabilisers, gelling agents, and emulsifiers in the food, textile, and pharmaceutical industries [15]. Alginates have also been widely employed for immobilizing living cells and enzymes [6]. Commercial alginates are currently extracted from marine algae such as *Laminaria* and *Macrocystis* and also can be obtained from bacterial sources, namely *Azotobacter vinelandii* and various species of *Pseudomonas*. Although alginate production has been widely studied in the pathogenic bacterium *Pseudomonas aeruginosa*, the soil bacterium *A. vinelandii* appears more suitable for alginate production because this bacterium is nonpathogenic and can also produce alginate with contiguous sequences of L-guluronic acid residues, similar to the algal alginate [17]. The rheological and gel-forming properties of alginate depend on the molecular mass distribution and on the relative content of the two monomers [9]. Bacterial alginates generally have higher molecular mass than algal polymers. The molecular mass of algal alginates ranges from 48,000 to 186,000 Da [9], whereas values up to 2×10^6 Da have been found for the polymers produced by *A. vinelandii* [12].

Poly-β-hydroxybutyrate (PHB) content may comprise up to 70% of cellular dry weight in certain strains of *A. vinelandii* [1]. In a previous study [12], we reported that *A. vinelandii* (ATCC 9046) produced 4.5 g/l alginate and accumulated up to 50% (wt/wt) of PHB at the end of the fermentation, when the bacterium was grown under mild aeration conditions in shake flasks. For maximising alginate production, synthesis of PHB could constitute an undesirable waste of substrate [2]. As both PHB accumulation and alginate secretion are associated with the successful completion of the encystment process, which generates spherical, desiccation-resistant cells called cysts, it may thus be difficult to separate the synthesis of these polymers in the wild-type organism. It is possible, however, to block the synthesis of PHB by mutation, overcoming one of the obstacles for producing alginates by *A. vinelandii* [8]. Although previous reports revealed the advantages of several mutant strains of *A. vinelandii* [4,7], there are no reports in the literature regarding the alginate-producing capacity of *A. vinelandii* mutants affected in PHB biosynthesis. Conversely, there are data [8] on the effect of a mutation within the alginate biosynthetic cluster, which improves the production of PHB in *A. vinelandii*. Another possibility for increasing alginate production is to isolate deregulated mutants, which, by means of an overexpression of alginate biosynthetic genes, can overproduce this polysaccharide [3].

The mean molecular weight (MMW) of the alginate is strongly influenced by the culture conditions. Recently, our group [13] reported that under dissolved oxygen tension (DOT) of 3% of air saturation, in connection with low agitation speed (300 rpm), *A. vinelandii* produced an alginate with a high molecular weight (500,000 g/g mol), whereas a low-molecular weight (352,000 g/g mol) alginate was isolated from cultures conducted at 700 rpm at the same DOT (3%). This latter phenomenon was associated with the synthesis of an alginate lyase under high agitation speed.

In the present work, alginate production by mutant strains of *A. vinelandii*, altered in their PHB biosynthetic capacity and in the regulation of the *algD* gene, was evaluated in batch culture under DOT-controlled conditions, and the molecular weight distribution (MWD) of the polysaccharide produced was determined.

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Materials and methods

Microbial strains

A. vinelandii strains used were the highly mucoid wild-type strain ATCC 9046 [3]; DS268, a derivative of the nonmucoid strain UW136 carrying a mini-Tn*5* insertion within the *phbR* gene, encoding a transcriptional regulator responsible for the activation of *phbBAC* biosynthetic operon and whose inactivation causes a reduction in PHB synthesis [14]; AT268, an ATCC 9046 derivative containing the mini-Tn*5* insertion of DS268 (this work); WI12, a derivative of ATCC 9046 that contains a transcriptional fusion of *algD* (coding for the GDP mannose dehydrogenase) with the reporter gene *lacZ* [3]; RC26, a WI12 derivative containing a mini-Tn*5* *luxAB* insertion (called *muc26* mutation) in an intergenic region 55 nt (nucleotides) downstream *recG* (this work); CNT26, an ATCC 9046 derivative that carries the *muc26* mutation; and DM, an AT268 derivative containing the *muc26* mutation (this work). *Escherichia coli* SM10 (λpir)/pUT-mini-Tn*5* *luxAB* [5] was used as the donor strain for the mini-Tn*5* mutagenesis and *E. coli* DH5α was used as host for plasmids in all cloning experiments. *A. vinelandii* strains were maintained by monthly subculture on Burk's agar slopes and stored at 4°C. *E. coli* strains were grown at 37°C in LB medium.

Culture medium and growth conditions

A. vinelandii strains were grown in a modified Burk's medium [12] containing 20 g/l sucrose and 3 g/l yeast extract. Cultures were carried out in a 1.5-l stirred tank, keeping pH constant at 7.2 by automatic addition of NaOH (2 N). DOT was measured with a polarographic oxygen probe (Ingold, Ingold Electrodes, Wilmington, MA, USA) and it was controlled at 3% (with respect to air saturation) by varying the proportions of nitrogen and oxygen in the inflowing gas, through two mass flow controllers and using a proportional control algorithm [13]. The gas mixture was sparged at a constant total flow rate of 800 SSCM at 29°C and agitated at 300 rpm. Samples of 20 ml were withdrawn every 6 h for analytical determinations.

Nucleic acid procedures

DNA isolation, digestion, cloning, and Southern blotting were carried out by standard procedures [16]. β-Galactosidase was determined as described by Miller [11]. The Gene Bank/EMBL accession number for the sequence reported in this paper is AY 116079.

Transposon mutagenesis

Transposon mutagenesis was carried out as described by De Lorenzo [5] with a pUT derivative containing the mini-Tn*5* *luxAB* transposon. This plasmid was transferred by conjugation into *A. vinelandii* strain WI12. Mating was done on Burk's sucrose–LB medium plates. The cells were resuspended in 10 mM $MgSO_4$ and plated on Burk's sucrose plates and tetracycline-resistant derivatives were isolated.

Analytical determinations

Alginate and PHB concentrations were determined as described before [12]. The viscosity of the culture broth was measured using a cone/plate viscometer (Wells-Brookfield LVT, Series 82198, Brookfield Engineering Laboratories, Stoughton, MA, USA). All determinations were made at room temperature (25°C) and 6 rpm using cone CP-52, which, according to the instrument manufacturer, corresponds to a shear rate of 12 s^{-1}.

Characterization of the alginate

The alginate MWD was estimated by gel permeation chromatography (GPC) with a serial set of Ultrahydrogel columns (UG 500 and 2000; Waters, Milford, MA, USA), using a conventional HPLC system with a differential refractometer detector. The columns were calibrated by a standard calibration method using pullulans of *Aureobasidium pullulans* as standards of molecular mass [12]. The acetyl content of the various alginates, expressed as acetate content, was determined as described elsewhere [10], using β-D-glucose pentaacetate (Sigma, Sigma-Aldrich Química, Toluca, México) as standard.

Reproducibility of experiments

The results reported are the average of at least three independent determinations. The maximum standard error of alginate and PHB determinations was 10%. Viscosity, MMW, and acetylation degree exhibited standard errors between 10% and 15%.

Results and discussion

Isolation of RC26

In order to identify genes whose inactivation could increase the transcription of alginate biosynthetic genes and consequently improve alginate production, we carried out a random mini-Tn*5* *luxAB* Tc^r mutagenesis of WI12 strain, which contains *algD-lacZ* gene fusion. After mutagenesis, a mutant strain called RC26, which exhibited a bluer colour than WI12 on BS plates containing X-gal, was isolated. Quantification of β-galactosidase activity at 24 h in 250-ml Erlenmeyer flasks, containing 50 ml of Burk's sucrose medium, showed a 1.9-fold increase in *algD* transcription in the RC26 strain (9.5±0.5 U) with respect to WI12 (5.0±0.4 U).

Cloning and DNA sequence

In order to identify the transposon-inactivated locus in strain RC26, a 6.8-kb *Pst*I DNA fragment containing the mini-Tn*5* insertion (5.2 kb) was cloned into pBluscript KS producing plasmid pC26. This plasmid was used to identify by hybridization the cosmid clone pSM4488, derived from an *A. vinelandii* genomic library. A 1.6-kb *Pst*I DNA fragment from this cosmid, corresponding to the 6.8-kb fragment of pC26, was subcloned into pBluescriptKS, producing plasmid pMX63. Sequence analysis of this DNA fragment revealed two open reading frames, one of them showing high similarity with the *recG* gene of *P. aeruginosa*, coding for DNA helicase [18], and an ORF downstream of *recG*, showing a striking similarity with gene PA 5346 of *P. aeruginosa* [18] whose function is unknown. We named this gene *muc26*. The sequencing across the transposon insertion junction showed that mini-Tn*5* insertion was within the intergenic region, 55 nucleotides downstream *recG* and 22 nucleotides upstream the ATG of *muc26*. To determine if the increase in *algD* transcription was due to a polar effect on *muc26*, we generated a Gm resistance gene insertion into the *Xho*I site of this gene and introduced this mutation in the WI12 strain by gene replacement. The resulting phenotype with respect to *algD* transcription was tested on BS plates with X-gal. This mutant also showed an *algD*-increased transcription (data not shown), confirming that the phenotype of RC26 was due to the polar effect of the *muc26* insertion.

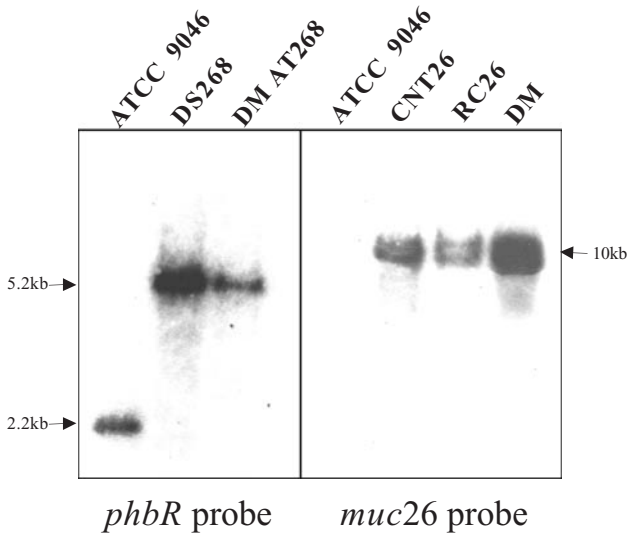

Figure 1 Southern blot hybridization analysis of the mini-Tn*5* insertions on the *phbR* and *muc26* genes in different *A. vinelandii* strains. For the *phbR* gene analysis, a 2.2-kb *Pst*I fragment containing this gene was used as probe. The DNA were digested with *Xho*I. For the *muc26* insertion, the tetracycline resistance gene contained in the miniTn*5* *lux*ABTc was used as probe and the DNA were digested with *Pst*I.

As WI12 (the parental strain of RC26) has a diminished alginate synthesis due to *algD-lacZ* insertion, we transferred the *muc26* mutation to the wild-type strain ATCC 9046. This was done by gene replacement using plasmid PC26, containing a *Pst*I DNA fragment with the mini-Tn*5* insertion from strain RC26 and unable to replicate in *A. vinelandii.* The resultant strain was named CNT26 and the replacement was confirmed by Southern blot analysis (Figure 1).

Construction of mutant AT268

In order to construct an ATCC 9046 derivative with a *phbR* mutation that reduced PHB synthesis, DNA from mutant DS268, an UW136 derivative containing a mini-Tn*5* insertion on the *phbR* gene [14], was transformed into ATCC 9046. A kanamycin-resistant transformant named AT268 was isolated and the gene replacement was confirmed by Southern analysis (Figure 1).

DM mutant construction

To generate a strain containing the *phbR* and *muc26* mutations, plasmid pC26 was introduced into AT268 strain by transformation. A transformant selected on a BS plate containing tetracycline was named DM and was confirmed by Southern blot analysis to carry the *muc26* insertion (Figure 1).

PHB and alginate production

In order to evaluate the effect of the *phbR* and the *muc26* mutations on alginate production, we tested CNT26, AT268 strains, and its derivative DM. As shown in Figure 2, mutants AT268 and DM accumulated a lower concentration of PHB than that found in the parental strain under the same culture conditions. The PHB content was 2% (wt/wt) in the mutant DM, whereas in the ATCC 9046, PHB comprised up to 46% (wt/wt) of the cell dry weight.

Unexpectedly, alginate production by all the mutants was lower than that obtained with the wild type. A maximum alginate concentration of 2.6 g/l was obtained with the mutant strains, whereas with the wild type, the alginate concentration was 3.5 g/l (Table 1). These data revealed that the decrease in PHB accumulation and/or the increase in the transcription of gene *algD* did not improve alginate production in the two mutants under the conditions tested and suggest that regulation of the carbon flux is

Table 1 Concentration and chemical characteristics of the alginate produced by different strains of *A. vinelandii* grown at 3% of DOT and 300 rpm

Strain	Alginate[a] (g/l)	MMW[b] (MDa)	PI[c]	Acetylation degree (%)
ATCC 9046	3.5	0.80	3.1	3.6
AT268	2.6	0.85	1.9	3.3
CNT26	2.6	0.50	1.7	N.D.[d]
DM	2.6	4.0	1.3	2.6

[a]Maximal concentration.
[b]Mean molecular weight.
[c]Polydispersity index.
[d]Not determined.

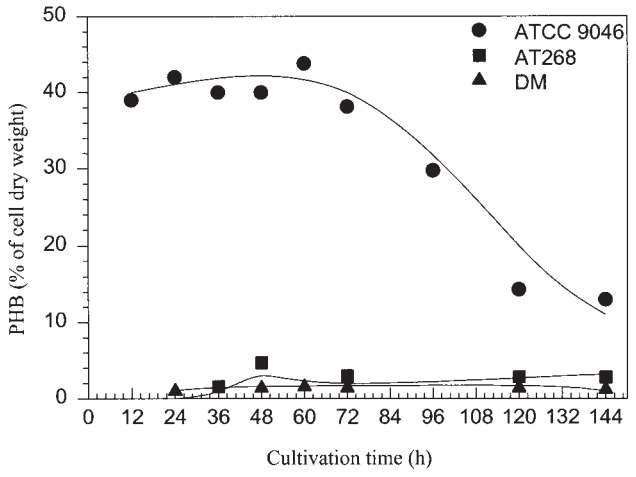

Figure 2 Evolution of intracellular PHB of cultures conducted in shake flasks with three strains of *A. vinelandii*.

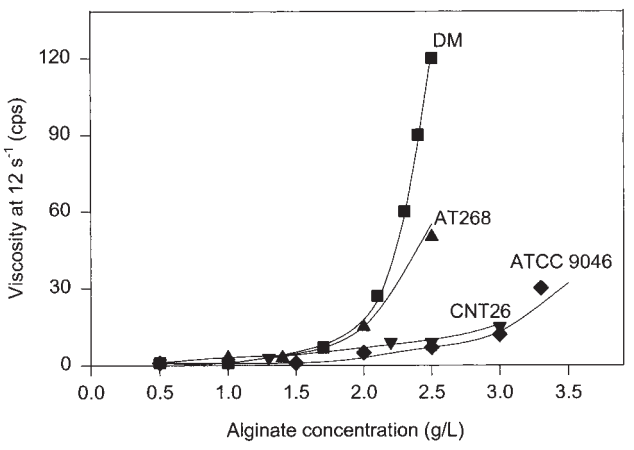

Figure 3 Viscosity of the culture broth as a function of alginate concentration for the different mutant strains used in this work.

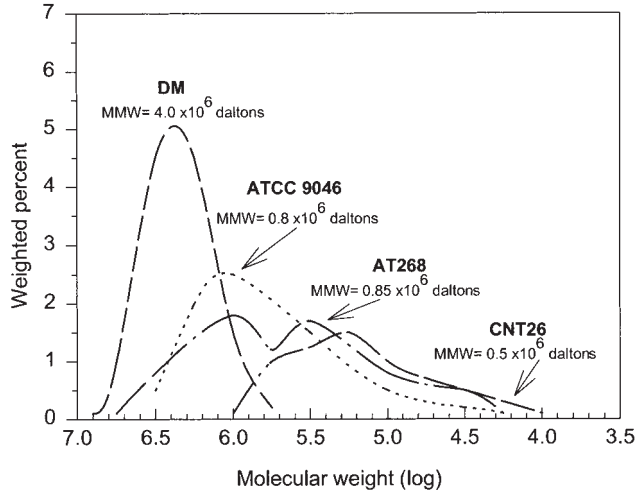

Figure 4 Molecular weight distributions of alginates isolated from cultures of four strains of *A. vinelandii*.

very complex and sucrose consumed is not necessarily diverted to alginate biosynthesis.

Although we cannot rule out the possibility of a second mutation during the construction of CNT26 and DM strains (generated independently), we believe that the negative result on alginate production could be due to the fact that the growth conditions used for alginate production were very different from the conditions used for isolation and testing of RC26 (1.5-l stirred tank, constant pH, 3% DOT, *versus* Petri dishes and Erlenmeyer flasks).

Characterization of the polymer

Unexpectedly, the most interesting difference between the strains was found in the broth viscosity. As shown in Figure 3, the viscosity (at the same alginate concentration) produced by mutants DM and AT268 was higher than the viscosity exhibited by the polymer obtained using the wild-type (ATCC 9046) and mutant CNT26. For example, the viscosity at the given polymer concentration (i.e., 2.5 g/l) of the alginate isolated from cultures with the DM mutant was 14-fold higher than the viscosity obtained with the wild-type strain. If compared with the viscosity obtained (at the same polymer concentration) with the algal alginate, the increase would be 12-fold [12]. From a practical point, this means that, to reach the same viscosity, a lower amount of alginate (produced by the DM mutant) would be required with respect to that needed when the algal alginate or the alginate produced using the wild-type strain was used. This could be especially interesting in particular applications of alginate such as thickeners in the food industry. As will be shown, these results are in line with the molecular weight of the alginates isolated from the culture broths.

Figure 4 shows the MWD of samples of alginate obtained from cultures with the different strains. Mutant DM produced an alginate with a very high molecular mass and low polydispersity (4.0×10^6 Da and polydispersity index, PI=1.3). In contrast, the maximum molecular mass (MMM) of alginate isolated from cultures carried out with mutants AT268 and CNT26, as well as with the wild type, was considerably lower (0.85×10^6, 0.5×10^6, and 0.8×10^6 Da, respectively). As far as we know, the molecular weight exhibited by the alginate produced by the DM mutant is the highest ever reported for a bacterial alginate. This value is considerably higher than that reported by our group for a commercial alginate from *Macrocystis pyrifera* (1.1×10^6 Da) and that of the alginate produced by *A. vinelandii* in shake flasks (2×10^6 Da) [12], determined using the same method used in this work.

The degree of acetylation of alginates produced by the mutant DM was lower than those determined in the alginate isolated from the cultures carried out with the wild strain (ATCC 9046) and mutant AT268 (Table 1). For alginate obtained with the DM strain, the content of acetyl residues was 2.6%, whereas in the wild type, the acetylation degree was 3.6% (Table 1). It is possible that alginate with lower acetyl content would produce stronger gels compared to polysaccharides with high acetylation degree, as it is known [17] that the alginate with a higher content of acetyl groups shows a drastic reduction in ion binding (mainly with divalent cations).

The higher molecular weight of the alginate produced by mutant DM (with respect to the wild strain and AT268 mutant) is not due to single mutations affecting the transcription levels of the alginate biosynthetic gene *algD* or to PHB biosynthesis, as the polymer produced by mutant CNT26 exhibited a MMM of 500,000 Da under the same conditions (Figure 4). Although no conclusive explanation can be given, the observed effect is an indication of the complexity of regulation of the molecular weight of the alginate produced by *A. vinelandii*. Further studies will be needed to elucidate in detail how the molecular weight of the polymer is regulated.

Conclusions

Alginate production and MWD of the polysaccharide synthesized by mutant strains of *A. vinelandii*, altered in their PHB biosynthetic capacity and in the regulation of the *algD* gene, were evaluated; unexpectedly, alginate production by the mutants was lower than that obtained with the wild type. However, a high viscosity and high molecular weight were obtained with the DM mutant. Although the reasons for this behaviour are not clear at the molecular level, from a practical point of view, the use of mutant DM would allow the production of an alginate with a very high molecular weight and a low acetylation degree, which could be particularly convenient in specific applications as a gelling agent and thickener in the food and pharmaceutical industries.

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