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New insights into *Pseudomonas fluorescens* alginate biosynthesis relevant for the establishment of an efficient production process for microbial alginates

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ABSTRACT

Alginate denotes a family of linear polysaccharides with a wide range of industrial and pharmaceutical applications. Presently, all commercially available alginates are manufactured from brown algae. However, bacterial alginates have advantages with regard to compositional homogeneity and reproducibility. In order to be able to design bacterial strains that are better suited for industrial alginate production, defining limiting factors for alginate biosynthesis is of vital importance. Our group has been studying alginate biosynthesis in *Pseudomonas fluorescens* using several complementary approaches. Alginate is synthesised and transported out of the cell by a multiprotein complex spanning from the inner to the outer membrane. We have developed an immunogold labelling procedure in which the porin AlgE, as a part of this alginate factory, could be detected by transmission electron microscopy. No time-dependent correlation between the number of such factories on the cell surface and alginate production level was found in alginate-producing strains.

Alginate biosynthesis competes with the central carbon metabolism for the key metabolite fructose 6-phosphate. In *P. fluorescens*, glucose, fructose and glycerol, are metabolised via the Entner-Doudoroff and pentose phosphate pathways. Mutational analysis revealed that disruption of the glucose 6-phosphate dehydrogenase gene *zwf-1* resulted in increased alginate production when glycerol was used as carbon source. Furthermore, alginate-producing *P. fluorescens* strains cultivated on glucose experience acid stress due to the simultaneous production of alginate and gluconate. The combined results from our studies strongly indicate that the availability of fructose 6-phosphate and energy requires more attention in further research aimed at the development of an optimised alginate production process.

Contents

Abbreviations: M, β-D-Mannuronic acid; G, α-L-Guluronic acid; EDP, Entner-Doudoroff pathway.

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1. Introduction

Alginates are industrially and medically widely used linear polysaccharides composed of the two monomers β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G), bound together by 1,4-linkages. In contrast to most other polysaccharides, the monomers in alginates are not organised in repeating units and almost all samples can be considered as a collection of heterogeneous molecules. The molar ratios between the two monomers and their sequential distributions vary widely and it is common to describe the monomer sequence along the polymer chains as consisting of varying frequencies of M-blocks, G-blocks and MG-blocks [1]. Each of these block types consist of contiguous stretches of their respective monomers, the length of these stretches varying from two to several hundreds. G-blocks are of great significance in the applied (and also biological) context, since they can mediate crosslinking of alginate molecules via binding of various divalent cations, leading to the formation of extensive polymer networks visible as macroscopic gels [2]. The properties of these gels can be varied by using different alginate types, enabling control of porosity, swelling and stiffness [3]. The gels can for example be used to encapsulate living cells, and they can easily be dissolved by simply adding chelators that bind the crosslinking cations. Alginates are also used as viscosifiers and emulsifiers for various purposes. For these applications, polymer chain lengths are of critical importance [2].

The commercial uses of alginates are currently entirely based on harvesting brown seaweeds, but the polymer is also produced by bacteria of the genera *Pseudomonas* and *Azotobacter*. So far, *Pseudomonas* alginates have not been reported to contain G-blocks, while such structures are abundant in polymers from seaweeds and *Azotobacter*. In all known alginate-producing bacteria, M-residues are converted to G after polymerisation by a periplasmic mannuronan C5-epimerase, but the *A. vinelandii* genome encodes seven additional and evolutionarily related mannuronan C5-epimerases that are secreted to the outer environment. Six of these are capable of introducing G-blocks of varying lengths into the polymer, enabling the production of the corresponding types of alginates by this organism [4].

2. Alginate biosynthesis in *Pseudomonas* spp.

In all alginate-producing bacteria investigated, a membrane-spanning protein complex consisting of six different proteins is necessary for polymerisation and secretion of the polymer (Fig. 1). The co-polymerase Alg44 has been shown to interact with the polymerase Alg8 as well as with other proteins in the complex [5,6]. Alg44 is only active when a dimer of c-di-GMP is bound, providing a post-translational regulation of alginate biosynthesis [7,8]. The corresponding genes are expressed from one main promoter in alginate-producing *Pseudomonas* spp. This operon also encodes proteins required for alginate modification and transport (AlgKEGXLIJF), and two of the three proteins needed for synthesis of the alginate precursor GDP-mannuronic acid from fructose 6-phosphate, AlgA and AlgD (Fig. 1). The third protein necessary for precursor biosynthesis, AlgC, is located in a separate operon. AlgG, AlgX, AlgK and AlgE are all required for transport of the nascent alginate chain through the periplasmic space and out of the cell. Moreover, AlgG epimerises some of the M-residues to G [9,10] and AlgX adds acetyl groups to the O-2 or O-3 hydroxyl groups of some of the M-residues [11]. The alginate operon also encodes an alginate lyase, AlgL, which removes any alginate molecules aberrantly localised in the periplasm [12]. AlgF, AlgI and AlgJ are required to transport an acetyl moiety to the alginate acetylase AlgX. Absence of AlgF, AlgI or AlgJ results in synthesis of non-acetylated alginate, while the other nine proteins encoded by the alginate operon are all necessary for alginate production.

Expression of the alginate biosynthetic genes depends on the sigma factor AlgU and is inhibited by the anti-sigma factors MucA and MucB that bind to and sequester AlgU (Fig. 1). It has been shown that some alterations in the cell wall lead to a proteolytic cascade that inactivates MucA and releases AlgU [13]. Furthermore, deletion of *mucA* results in constitutive alginate production in *Pseudomonas* spp. However, in *Pseudomonas aeruginosa mucA* mutants, suppressor mutations that abolish alginate production frequently arises [14]. Transcription of the *algU-mucA-mucB* operon also depends on AlgU. In *P. aeruginosa* and *A. vinelandii*, the operon further encodes the regulatory genes *mucC* and *mucD*. However, *mucC* is lacking in both *P. fluorescens* and *Pseudomonas syringae,* and in the latter it has been shown that *mucD* is transcribed from an AlgU-independent promoter [15].

3. Towards industrial production of bacterial alginates

Manufacture of alginates from seaweeds has the disadvantage that these polymer products do not always display the structural composition desired for specific applications, and environmental concerns related to seaweed harvesting have also been raised. Both problems can potentially be solved by producing alginates from bacteria cultivated in bioreactors. The factors controlling alginate production and structure in these organisms are now so well known that it should be possible to modify the bacteria in such a way that nearly any desired alginate type could be produced, possibly by generating a collection of different strains in which each produces a particular alginate type of interest. For microbial production to become commercially viable, it will obviously be of critical importance to develop a process that enables production at the lowest possible cost. This has motivated us to study the factors determining alginate production rates and yields in bacteria.

In our studies, we have utilised two wild type strains of *P. fluorescens*, NCIMB10525 and SBW25 [16], neither of which produces alginate under standard laboratory conditions. Strain Pf201 is a constitutive alginate-producing strain obtained by chemical mutagenesis of NCIMB10525 [17]. A *mucA*$^-$ derivative of strain SBW25 has been constructed and is also a stable alginate producer [18]. Furthermore, the inducible *Pm*-promoter from the *Pseudomonas putida* Tol-plasmid has been successfully utilised to express single genes on transposons and to replace the promoters controlling alginate biosynthetic genes in *Pseudomonas* strains [9,19].

P. fluorescens has proved to be an advantageous model system for studying alginate biosynthesis. The bacterium is non-pathogenic and stable mutants producing copious amounts of the polymer have been obtained [9,18]. In batch fermentations, up to 17 g/l alginate has been reported after about 48 h fermentation, using fructose or glycerol as carbon source. On glucose, the yield was around 13 g/l. The yield on carbon basis was 33, 40 and 43% when glucose, fructose and glycerol, respectively, were used as carbon sources [17]. In chemostat fermentations, about 70% of the imported fructose or glycerol was converted to alginate by *P. fluorescens* SBW25 *mucA* [18].

4. Establishing a method for visualisation of the alginate biosynthetic complexes

Recently we have studied the importance of the expression level of the *alg* gene cluster in maximising alginate production. This is clearly relevant since the promoter upstream of the *alg* operon (the *algD* promoter) is tightly regulated and essentially inactive in most wild type *Pseudomonas* strains, resulting in

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