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EXTRACELLULAR POLYSACCHARIDES

REVIEW

Gellan gum biosynthesis in *Sphingomonas paucimobilis* ATCC 31461: Genes, enzymes and exopolysaccharide production engineering

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The commercial gelling agent, gellan, is an extracellular polysaccharide (EPS) produced by *Sphingomonas paucimobilis* ATCC 31461. In recent years, significant progress in understanding the relationship between gellan structure and properties and elucidation of the biosynthesis and engineering of this recent product of biotechnology has been made. This review focuses on recent advances in this field. Emphasis is given to identification and characterization of genes and enzymes involved, or predicted to be involved, in the gellan biosynthetic pathway, at the level of synthesis of sugar-activated precursors, of the repeat unit assembly and of gellan polymerization and export. Identification of several genes, biochemical characterization of the encoded enzymes and elucidation of crucial steps of the gellan pathway indicate that possibilities now exist for exerting control over gellan production at any of the three levels of its biosynthesis. However, a better knowledge of the poorly understood steps and of the bottlenecks and regulation of the pathway, the characterization of the composition, structure and functional properties of gellan-like polymers produced either by the industrial strain under different culture conditions or by mutants are still required for eventual success of the metabolic engineering of gellan production.
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The commercial bacterial exopolysaccharide gellan: composition and functional properties

Many Gram-negative bacteria synthesize and excrete extracellular polysaccharides (EPS), which may have diverse biological roles, as virulence factors in plant and animal pathogens, signalling molecules in bacteria–plant interaction and contributing to cell protection from environmental aggression [34]. Some of these EPS are potential or accepted products of biotechnology. This is the case of the commercial gelling agent gellan, produced by large-scale submerged fermentation of *Sphingomonas paucimobilis* ATCC 31461 with a typical yield of gellan from glucose of 40–50% [46]. It has approval in the US and EU for food use as a gelling, stabilizing and suspending agent, either alone or in combination with other hydrocolloids [6,41]. In its native form, gellan is a linear anionic heteropolysaccharide based on a tetrasaccharide repeat unit composed of two molecules of D-glucose (D-Glc), one of L-rhamnose (L-Rha) and another of D-glucuronic acid (D-GlcA) (Figure 1) [20,33]. The native gellan is partially esterified: the 1,3-D-Glc residue can be linked to L-glycerate at C-2 and/or to acetate at C-6 (Figure 1) and there is 1 mol of glycerate and 0.5 mol of acetate per repeating unit [23]. Acyl substituents drastically affect the rheology of the gels formed with various cations; chemical deacylation of the native form results in a change from soft, elastic thermoreversible gels to harder, more brittle gels [6].

Structure and rheological characterization of gellan-related polysaccharides differing essentially in the content of acetate and/or glycerate confirmed both predictions from X-ray studies and results from rheological studies on chemically deacylated gellan, i.e., the glycerate substituent which is responsible for significant changes in rheology observed upon deacylation [21]. These gellan-like polymers are produced by mutants obtained by exposure of cultures of the producing strain to chemical mutagens and to environmental stress, in particular to antibiotic or copper stress,

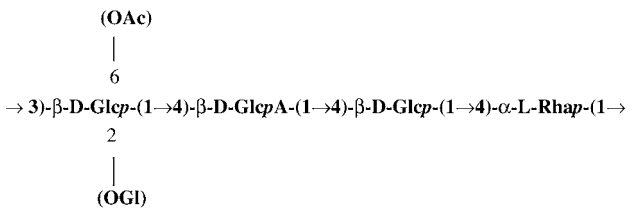

Figure 1 Repeating unit of the exopolysaccharide gellan produced by *S. paucimobilis* ATCC 31461. In the native polymer, O-acetyl (OAc) is present at 0.5 mol per repeating unit and O-glyceryl (OGl) at 1 mol per repeating unit. D-Glc, D-glucose; D-GlcA, D-glucuronic acid; L-Rha, L-rhamnose.

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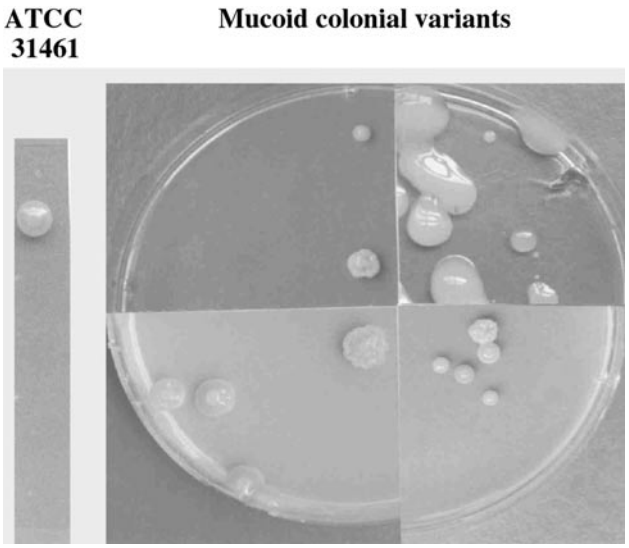

Figure 2 Mucoid colonial variants of *S. paucimobilis* ATCC 31461 obtained by chemical mutagenesis or exposure to antibiotic or copper stress. These mutants produce gellan-related polysaccharides with different substitution patterns.

followed by selection based on the distinct mucoid morphology of their colonies [21,39], as exemplified in Figure 2. The small number of variants of the gellan structure compared contained both glycerate and acetate in different levels or only acetate substitution. A gellan variant with acetate and lacking glycerate was examined for the first time [21] because this combination is not possible to obtain by chemical deacylation. The characterization of the different structure and properties of gellan produced by the industrial strain on different carbon sources and growth media [16] and by a larger number of gellan-like polysaccharides may provide further clues to understand the relationship between gellan structure and properties — a prerequisite to success in this polysaccharide engineering. Another prerequisite to manipulate the gellan pathway is elucidation of the molecular biology, biochemistry and physiology of its biosynthesis in the producing strain, *S. paucimobilis* ATCC 31461. Recent advances in these aspects of the molecular microbiology of gellan production are summarized below.

Genes and enzymes involved in gellan biosynthesis

Genes and enzymes involved in the formation of nucleotide sugar precursors

The biosynthetic pathway up to the sugar precursors: The biosynthetic pathway leading to the nucleotide sugar precursors UDP-D-Glc, dTDP-L-Rha and UDP-D-GlcA, which are the donors of monomers for biosynthesis of the tetrasaccharide unit in gellan [26], is shown in Figure 3. The identification, sequence analysis and biochemical characterization of genes/enzymes involved in catalysis of two steps in this pathway were already carried out: (1) the *pgmG* gene, encoding a phosphoglucomutase (PGM; EC 5.4.2.2) which catalyses the interconversion of glucose-6-phosphate (G6P) into glucose-1-phosphate (G1P); (2) the *ugpG* gene, encoding a G1P uridylyltransferase [or UDP-glucose pyrophosphorylase (UGP; EC 2.7.7.9)] which catalyses the reversible conversion of G1P and UTP into UDP-D-Glc. Among the gellan biosynthetic enzymes, the PgmG protein plays a pivotal role, presumably being an ideal target for metabolic engineering. Indeed, this enzyme catalyses a step representing a branch point in carbohydrate metabolism; G6P enters catabolic processes to yield energy and reducing power, whereas G1P is a precursor of sugar nucleotides that are used by cells in the synthesis of various polysaccharides. The enzyme UgpG is involved in the synthesis of the gellan-activated precursor UDP-glucose, which gives rise to another gellan precursor, UDP-glucuronic acid, by the step catalysed by UDP-glucose dehydrogenase. These two activated sugars are donors in the biosynthesis of gellan tetrasaccharide repeat unit.

PGM: the gene and the enzyme: The *pgmG* gene from *S. paucimobilis* ATCC 31461 was cloned based on PCR amplification of a genomic fragment using degenerate primers from conserved sequences of phosphohexosemutase proteins in databases [47]. This gene (GenBank database; accession no. AF167367) encodes a 50-kDa polypeptide that, after purification to homogeneity, was proved to exhibit PGM and phosphomannomutase (PMM) activities [47]. This bifunctional protein, PgmG, is 83% identical to the Pgm of *Sphingomonas* S7 [42], a strain that produces a gellan-related polysaccharide, both known as "sphingans" after the common genus to which the producing strains belong. PgmG is 53–37% identical to other PGM/PMM proteins from Gram-negative species, including the *Pseudomonas aeruginosa* AlgC required for alginate biosynthesis [57]. A lower level of homology was also noted with a number of phosphoglucosamine mutases [47]. Multiple amino acid alignments of the predicted PgmG amino acid sequence with the primary structures of other proteins with PGM and/or PMM activities, from prokaryotic and eukaryotic organisms, confirmed the three conserved domains found among hexosephosphate mutases, boxes I, II and III [53]. Box I, with the

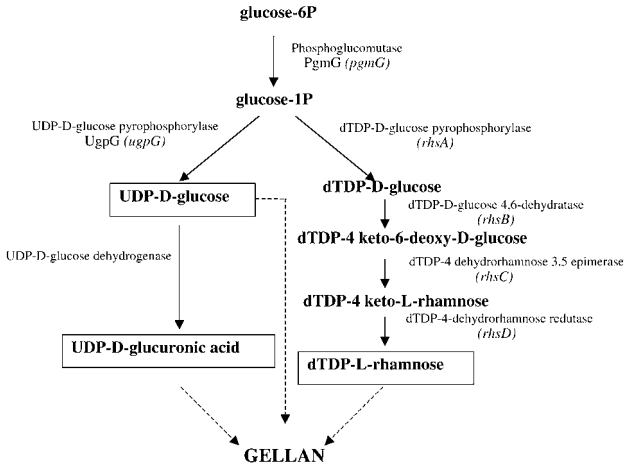

Figure 3 Pathway leading to the nucleotide sugar precursors, UDP-D-glucose, UDP-D-glucuronic acid and dTDP-L-Rhamnose, involved in gellan gum biosynthesis. The genes that are predicted or proved to encode some of the enzymes are in parenthesis. Abbreviations: PgmG, phosphoglucomutase (*pgmG*); UgpG, UDP-D-glucose pyrophosphorylase (*ugpG*); TGP, dTDP-D-glucose pyrophosphorylase; UGD, UDP-D-glucose dehydrogenase; TRS, dTDP-L-rhamnose enzyme system [dTDP-D-glucose 4,6-dehydratase (*rhsB*)+dTDP-4 dehydrorhamnose 3,5-epimerase (*rhsC*)+dTDP-4 dehydrorhamnose reductase (*rhsD*)].

conserved residues [T-X-S-H-N-P], corresponds to the active site region, in which the serine residue (S_{104} for *S. paucimobilis* PgmG) is phosphorylated during the catalytic action of hexose-phosphate mutases [8,22,28,38]. Box II [D-X-D-X-D-R] contains a magnesium-binding site [8]. Box III [G-E-[ME]-S] may be responsible for interaction with the substrate [4,38]; PgmG exhibits in box III the methionine (M) residue, typical of the subclass of bifunctional enzymes, with PGM and PMM activities, instead of the glutamic acid (E) residue, present in the subclass of specific PGM [4,47]. Consistent with this sequence analysis of the *pgmG*, the biochemical characterization of the PgmG protein confirmed its bifunctionality, although with a marked preference for G1P, compared with mannose-1-P (M1P) (the catalytic efficiency, based on the K_{cat}/K_m ratio, was about 50-fold higher for G1P than it was for M1P and the estimated apparent K_m values for G1P and M1P are high, 339 and 1270 μM, respectively) [47].

Although the catalytic efficiency of *S. paucimobilis* PgmG was higher for G1P than it was for M1P, the *pgmG* gene cloned into pMMB66(EH) led to the recovery of alginate biosynthetic ability when introduced into a *P. aeruginosa* mutant with a defective *algC* gene [57]. This observation clearly indicates that PgmG protein can convert mannose-6-P into M1P efficiently in the initial steps of alginate biosynthesis and, together with the other results reported, suggests that PgmG may convert G6P into G1P in the gellan pathway.

UGP: the gene and the enzyme: The *ugpG* gene, encoding the UGP activity, was identified in a chromosomal region distinct from the *pgmG* gene *locus* by screening the *S. paucimobilis* ATCC 31461 genomic library for ability to complement the deficiency of an *Escherichia coli galU* mutant by restoring growth on galactose with medium acidification [50]. The deduced amino acid sequence encoded by the *ugpG* sequence (GenBank database; accession no. AF461178) was compared with the data deposited in the GenBank and showed a high level of homology with the Ugp enzymes of a variety of EPS-producing bacteria: 66% identity with the UDPGP from *Zymomonas mobilis* [44] and 59% identity with the Ugp enzyme from *Gluconoacetobacter xylinus* [24] and *Agrobacterium tumefaciens* [18]. UgpG is highly homologous (69% identity) to a putative Ugp protein of *Novosphingobium aromaticivorans* (previously *S. aromaticivorans*) (URL:http://www.jgi.doe.gov/JGI_microbial/html/nosphingobium/sphingo_homepage.html), but little or no similarity was found with eukaryotic enzymes with UGP activity, consistent with previous reports [10,15].

The predicted amino acid sequence of *S. paucimobilis* ATCC 31461 UgpG was compared with the primary structures of other UGPs from prokaryotic organisms available in the databases by using the CLUSTAL V alignment program [17]. Two regions (domains I and II) were highly conserved throughout the sequences compared. The N-terminal region (domain I) of UgpG protein exhibits the motif [G-X-G-T-R-X-L-P-X-T-K], which is highly conserved among the bacterial XDP-sugar pyrophosphorylases. This domain was postulated to constitute at least a portion of the activator-binding site [5,43] and represents a prototypic structure characteristic of the large family of bacterial XDP-sugar pyrophosphorylases [5]. Domain II, found among bacterial UGP, is also present in UgpG; this possesses a lysine residue (K_{192} for *S. paucimobilis* UgpG) located within the V-E-K-P motif, predicted to be essential in substrate binding or catalysis [43]. Furthermore, this motif, or a closely related sequence [A-E-K-P, K-E-K-P, Q-E-K-P or I-E-K-P] is found in many bacterial enzymes that catalyze synthesis of nucleoside diphosphate sugars from sugar-1-phosphates and nucleoside triphosphates [43]. Although UgpG and other prokaryotic UGP do not possess sequence homology with eukaryotic pyrophosphorylases, all these proteins contain the lysine residue predicted to be indispensable for enzyme function [7].

Enzyme assays using the purified UgpG fusion protein confirmed the UGP activity of UgpG, showing a typical Michaelis–Menten substrate saturation pattern with a very high affinity for UDP-glucose (K_m=7.5 μM) [25].

Genes and enzymes involved in the formation of gellan tetrasaccharide unit and in polymerization and export

The gellan cluster of genes: The gellan genes *pgmG* and *ugpG* do not map in the same *locus* and are not present in the cluster of genes involved in gellan synthesis recently identified [48]. The organization and sequence of the regions that have been examined so far within the gellan gene cluster (*gel* cluster) are highly similar to those present in the cluster of genes (*sps* cluster) required for the synthesis of the sphingan S-88, identified and fully sequenced by Yamazaki *et al* [56] (Figure 4). This EPS S-88 is produced by *Sphingomonas* S88 and shares a common structure with gellan gum and other sphingans, with a repeating unit composed of two molecules of D-Glc, one of D-GlcA and another of L-Rha or L-mannose and a molecule of L-Rha as a side group [56]. A homology search at the protein level revealed that the *gel locus* includes genes that encode enzymes involved in dTDP-L-Rha synthesis from G1P, in a four-step process encoded in the *sps* cluster by the *rhsACBD* genes; the presence of *rhsA* and *rhsD* homologous genes was confirmed in the gellan cluster, with *rhsC* and *rhsB* probably also present. The gellan cluster also includes glycosyltransferases involved in assembly of the repeating unit, and other proteins indispensable for gellan polymerization and export.

Genes and enzymes involved in the assembly of gellan repeat unit: Amino acid sequence similarity studies and biochemical characterization of one gene product (GelK) indicate that the gellan cluster includes genes encoding the glycosyltransferases essential for assembling the repeating tetrasaccharide unit

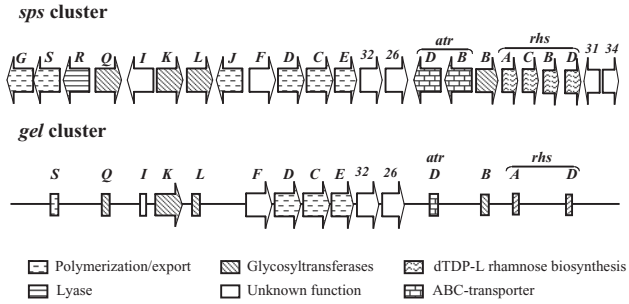

Figure 4 Schematic representation of the partially sequenced gene cluster required for biosynthesis of gellan by *S. paucimobilis* ATCC 31461 (*gel* cluster), compared with the cluster involved in the biosynthesis of S-88 by *Sphingomonas* S88 (*sps* cluster) (adapted from Ref. [56]). The predicted or experimentally proved biological function of the different genes (or group of genes) is indicated.

(Figure 4). They catalyse the sequential transfer of sugars from the appropriate sugar donor to an activated lipid carrier. The gellan *locus* includes *gelB*, homologous to *spsB* that encodes the priming transferase [36,37], a glucosyl-isoprenyl phosphate-transferase that transfers G1P from UDP-glucose to a C_{55}-isoprenylphosphate lipid carrier (PPL). Biochemical characterization of *gelK* (GenBank database; accession no. AF305842) revealed that it encodes a UDP-glucuronate, glucosyl-PPL β-1,4-glucuronosyltransferase, which catalyses the transfer of GlcA from UDP-glucuronic acid into the activated lipid-linked glucose [48]. The incorporation of radiolabelled GlcA from UDP-[^{14}C]GlcA, mediated by GelK crude extracts, was significantly increased in the presence of the glycolipid acceptor prepared from cells of *S. paucimobilis* or *E. coli* and this lipid anchor was the limiting factor in the enzyme assay [48]. Replacement of UDP-GlcA by UDP-glucose or UDP-galactose resulted in no sugar incorporation into the glycolipid fraction, consistent with the exquisite stereospecificity and precise stereochemistry of glycotransferases toward the substrate. GelK was not able to use synthetic glucosyl derivatives as acceptors, indicating that the pyrophosphate–lipid moiety is needed for enzymatic activity. GelK is presently both associated to plasma membrane and in the soluble form, being the membrane target region located at the N-terminal domain. Based on biochemical characteristics and sequence similarity studies, it was proposed that GelK is structurally related to the *E. coli N*-acetyl glucosaminyl transferase, MurG [48].

The other two glycosyltransferases required for gellan synthesis are possibly encoded by *gelL* and *gelQ* genes, also present in the gellan cluster (Figure 4) and probably involved in addition of the third and fourth sugars to the repeating unit [37]. However, there is no experimental evidence supporting this prediction.

Despite the crucial role that the substituent groups, acetyl and glyceryl (which decorate native gellan) have on rheological properties of the polymer, no information is yet available on the genes and enzymes that may catalyse this step of gellan biosynthesis or other aspects of the underlying biological process.

Genes and enzymes predicted to be involved in gellan polymerization and export: The mechanistic details of polymerization, chain-length determination and export of cell surface polysaccharides are still poorly understood. The information available refers mainly to the biosynthesis of *Salmonella* O-antigens (reviewed in Ref. [54]) and *E. coli* and *Streptococcus pneumoniae* capsular polysaccharides [13]. In the current model, the lipid-linked repeat units are transferred across the plasma membrane by the Wzx protein, using a mechanism that has yet to be established. Sequence similarities place Wzx in a family of polysaccharide exporter proteins designated as PST (*p*olysaccharide-*s*pecific *t*ransport) by Paulsen *et al* [35]. Polymerization appears to occur at the plasma membrane and is catalysed by the polymerase enzyme, Wzy, that uses as a substrate for polymerization the lipid-linked repeat units [13]. The polymerase, Wzy, is an integral membrane protein located in the cytoplasmic membrane, with 12 transmembrane segments and two large periplasmic loops [9]. While polymerization of Wzy-dependent O-antigens is regulated by a chain length-determining protein, Wzz, the length of groups 1 and 4 capsular antigens from *E. coli* is not influenced by Wzz [55]. Instead, the gene clusters for those capsules contain a gene (*wzc*) encoding a member of the PCP2a (*p*olysaccharide *co*polymerase) family [30]. The members of this family of proteins exhibit a large N-terminal hydrophilic region between two transmembrane domains, located on the periplasmic side of the cytoplasmic membrane [2], while the C-terminal region carries an ATP-binding domain, which was implicated in their tyrosine kinase autophosphorylation ability [11,19,31,32]. Although the process of tyrosine phosphorylation is still poorly understood, experimental evidences were obtained, implicating protein tyrosine phosphorylation in the negative regulation of capsular polysaccharide production in *St. pneumoniae* [31] and also colanic acid synthesis in *E. coli* [49]. Site-directed mutagenesis of specific tyrosine residues in the cytoplasmic domain of the *Sinorhizobium meliloti* protein ExoP (a member of the PCP2a family) resulted in an altered ratio of low-molecular-weight succinoglycan to high-molecular-weight succinoglycan [32], consistent with its role in the regulation of polysaccharide chain length.

Computational analysis as well as experimental evidence suggest that the sphingan clusters (Figure 4), responsible for biosynthesis of gellan by *S. paucimobilis* ATCC 31461 and EPS S-88 by *Sphingomonas* S88, encode homologues to proteins involved in polymerization/secretion of polysaccharides. In the sphingan S-88 biosynthesis, the products of genes *spsS* and *spsG* are homologous to Wzx and Wzy proteins, respectively [45]. A homologous region to *spsS* was also amplified by PCR using the *gel* cluster DNA region as the template, but no equivalent region to *spsG* was obtained by PCR amplification (Videira P, LM Moreira and I Sá-Correia, unpublished data), suggesting that this gene may be absent from the *gel* cluster.

All of these enzymes from Gram-negative bacteria belonging to the PCP2a family identified so far are encoded by a single gene. However, the equivalent peptides of the *sps* and *gel* clusters are encoded by two genes (*spsC* and *spsE* in *Sphingomonas* S88 and *gelC* and *gelE* in *S. paucimobilis* ATCC 31461). These two pairs of proteins, although related by sequence similarity and combined size to the known PCP2a proteins, are separated into two ORFs, which is a characteristic of the Gram-positive bacterial members included in family PCP2b [30]. Consistent with the predicted role of *gelC* and *gelE* genes, nonpolar mutants for these genes showed a drastic reduction in gellan production [29]. Experimental evidence indicates that GelE protein binds ATP, but no information is available about the possible tyrosine kinase autophosphorylation activity of GelE protein [29].

The cell surface polysaccharides are synthesized at the inner membrane and must be translocated to the cell surface. While protein translocation through the plasma membrane is well understood, virtually nothing is known about the translocation of cell surface polysaccharides across the outer membrane of Gram-negative bacteria. Recently, Drummelsmith and Whitfield [14] identified the protein Wza_{K30}, a member of the *o*uter *m*embrane *a*uxiliary (OMA) protein family [35] involved in the surface expression of the group 1 K30 capsular polysaccharide of *E. coli* EG9. This protein is a surface-exposed outer membrane lipoprotein that forms ring-like structures in the outer membrane reminiscent of the secretins of types II and III protein translocation systems [3,13]. Since a mutation in Wza_{K30} severely restricts the formation of the K30 capsular structure on the cell surface, this protein was proposed to form an outer membrane pore through which the K30 capsular antigen is translocated. The broad distribution of the OMA family homologues in diverse Gram-negative bacteria suggests a similar process for polysaccharide export. In *S. paucimobilis* ATCC 31461, the member of the OMA family is encoded by *gelD* (Figure 4) but there is no experimental evidence confirming its involvement in gellan export.

Environmental and genetic engineering of gellan production

Effects of environmental conditions on gellan biosynthesis

Although the production yields, composition, structure and properties of the gellan produced by *S. paucimobilis* ATCC 31461 are genetically determined, it is possible to influence these factors by modifying culture conditions such as temperature [27], oxygen transfer [12] and growth medium composition, in particular the carbon source [16] and the nitrogen source [51,52].

Gellan gum biosynthesis is temperature-dependent, with a maximal production yield at 20–25°C, which is well below the optimal range for growth (30–35°C) and that for maximal activities of gellan enzymes in the producing cells (30–35°C). In addition, the biopolymer synthesised at 20°C gives rise to solutions with maximal viscosity; unfortunately, there is no information on the chemical composition or the molecular mass of the polymers produced at the different temperatures. Despite the uncertainties of characteristics of biopolymers synthesised at various temperatures, it was proposed that a more rapid turnover of the carrier lipid, at temperatures causing higher specific growth rates, may lead to an earlier release of a polymer with a shorter chain length at optimal temperatures for growth [27]. According to this hypothesis, the carrier lipid available is preferably used for simultaneous synthesis of the peptidoglycan and lipopolysaccharide, limiting the level and size of the gellan produced, as for other EPS-producing bacterial systems [40].

Comparison of gellan biosynthesis by *S. paucimobilis* ATCC 31461 in a synthetic medium containing glucose or lactose (5–30 g/l) and in diluted sweet cheese whey indicates that alteration of the growth medium can markedly affect the polysaccharide yield, acyl substitution level, polymer rheological properties and susceptibility to degradation [16]. Cheese whey, an end-product of cheese production, is a nutrient-rich medium [with lactose (5%), lactic acid, proteins, fat, minerals and vitamins] whose disposal has been a concern to the dairy industry. The most desirable way of handling this waste is to utilize it as a substrate for production of useful products, and gellan gum can be considered one of them, with the simultaneous reduction of its biological oxygen demand [16]. Depression of gellan production from lactose compared with gellan production from glucose (approximately 30%) did not appear to occur at the level of synthesis of sugar nucleotides [16]. Lactose-derived biopolymers exhibited the highest total acyl content; the glucose and whey-derived gellans had similar total acyl contents but differed in their acetate and glycerate levels. Rheological studies revealed how the functionality of a gellan polysaccharide is affected by changes in acyl substitution; the viscosity values appeared to be directly related to the level of glycerate present [21]; the lactose-derived polymer, which had the highest total acyl content, yielded the lowest modulus.

A number of complex nitrogen sources support gellan production by *S. paucimobilis* ATCC 31461, increasing production yield when a complex nitrogen source is present instead of ammonium sulfate [51]. Both the nature and the concentration of the nitrogen source affect the gellan yield, being gellan- and biomass production-enhanced by yeast extract supplementation of a soy-based medium [52]. The role of fermentor hydrodynamics on gellan fermentation kinetics and the rheological properties of the culture broth are also important, increasing gellan production when oxygen transfer capacity is improved [12].

Genetic engineering of the gellan pathway

Perhaps the most exciting prospects for gellan modification and increasing production yield are found in genetic engineering. A few attempts to increase the relatively low conversion efficiency of gellan from glucose in *S. paucimobilis* ATCC 31461 (about 40–50%) [46] compared with the nearly 60–80% of sugar conversion into xanthan gum have been reported [1,46]. By random mutagenesis, synthesis of the gellan-competing poly-β-hydroxybutyrate synthesis was eliminated with no positive effect on the efficiency of gellan production [1]. By site-specific mutagenesis, the *zwf* gene encoding G6P-dehydrogenase was inactivated, envisaging diverting carbon flow toward gellan synthesis, apparently without the expected results [46].

Identification of a few genes and elucidation of crucial steps of the gellan pathway, as summarized above, indicate that possibilities now exist for exerting control over gellan production at any of the three levels of its biosynthesis — at the level of synthesis of sugar-activated precursors, of the repeat unit assembly and of gellan polymerization and export — by modifying expression of any of the individual gellan genes or of a group of these genes. Biosynthesis of gellan starts with the intracellular formation of the sugar nucleotides catalysed by PGM PgmG encoded by *pgmG* (Figure 5). PgmG is a key enzyme in the pathway and, apparently, an ideal target for metabolic engineering as it represents a branch point in carbohydrate metabolism; G6P enters catabolic processes to yield energy and reducing power whereas G1P is a precursor of all the sugar nucleotides that are used in the synthesis of gellan and other cell polysaccharides. Afterwards, the UDP-glucose-pyrophosphorylase, encoded by the *ugpG* gene, is directly involved in the formation of UDP-glucose, from G1P, and indirectly involved in the formation of another gellan activated precursor, UDP-glucuronic acid, derived from UDP-glucose through the biosynthetic step catalysed by UDP-glucose dehydrogenase (Figure 5). Synthesis of the sugar precursors is followed by synthesis of the repeat unit by sequential transfer of the sugar donors to an activated lipid carrier by committed glycosyltransferases. Among them is the protein encoded by the *gelK* gene, catalysing the step by which

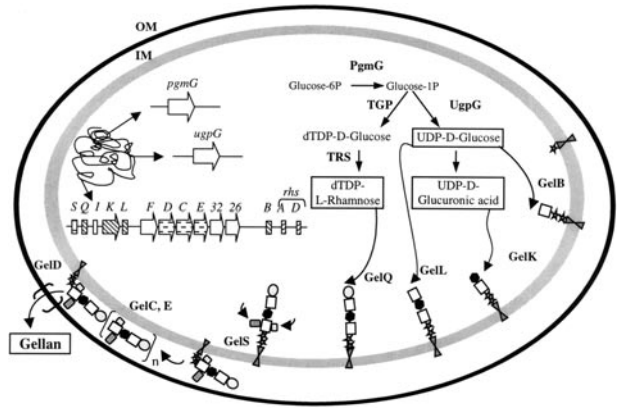

Figure 5 Schematic representation of identified or predicted genes and enzymes involved in gellan gum biosynthesis by *S. paucimobilis* ATCC 31461. Abbreviations: PgmG, UgpG, TGP, TRS (see legend of Figure 3); GelB, priming glucosyltransferase; GelK, glucuronosyltransferase; GelL and GelQ, putative glycosyltranferases; GelS, putative translocase; Gel D, C and E, proteins putatively involved in polysaccharide chain length regulation and secretion; OM, outer membrane; IM, inner membrane. (▱) Lipid carrier. (★) Phosphate. (□) Glucose. (◆) Glucuronic acid. (○) Rhamnose. (▭) Glycerate. (■) Acetate.

glucuronic acid is linked to the first lipid-linked glucose. *gelS* gene, which maps in the gellan *locus*, is predicted to encode a translocase involved in the last steps of polymer synthesis and export. Nucleotide sequence analysis and experimental evidence suggest that *gelC* and *gelE* genes in the *gel* cluster may be involved in the regulation of gellan chain length, with high impact on gellan properties. Metabolic engineering of the gellan pathway can now be attempted. Preliminary results indicate that augmentation of the expression of individual gellan biosynthetic genes, by increasing the number of *pgmG*, *ugpG* or *gelK* gene copies in recombinant plasmids, has apparently no positive effect on gellan productivity (Videira P, AR Marques, AM Fialho and I Sá-Correia, unpublished). However, the simultaneous increase of *pgmG* and *gelK* expression led to a 20% increase in the final concentration of gellan produced and to a polymer leading to aqueous solutions with higher viscosity. A similar result was reported before for genetic manipulation of the sphingan S-7 production in *Sphingomonas* S7 [42]; while a sixfold increase of PGM activity (by augmentation of the chromosomal gene copy with multiple copies of a plasmid carrying the cloned gene) had a negligible effect in glucose conversion, multiple biosynthetic genes from the S7 cluster, which code for assembly of the lipid-linked carbohydrate repeat unit and secretion of the polymer, caused a 20% increase in the yield from glucose and a larger increase in culture viscosity. This increased viscosity was associated with a decrease in the ratio of glucose to rhamnose compared with the original S-7 polymer [42]. In spite of recent advances in the elucidation of the gellan biosynthetic pathway, a better knowledge of the poorly understood steps and of the regulation and bottlenecks of the pathway is crucial to the eventual success of the metabolic engineering of gellan production.

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