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Influence of Deletions in the Apoemulsan Gene Cluster on *Acinetobacter venetianus* RAG-1 Polysaccharide Biosynthesis

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Apoemulsan is a biopolymer with potent emulsification activity, produced by *Acinetobacter venetianus* RAG-1 (RAG-1). The *wee* gene cluster is responsible for apoemulsan biosynthesis. The analysis of (i) a putative polysaccharide copolymerase mutant (Δwzc), (ii) a putative polymerase mutant (Δwzy), and (iii) an apoemulsan-deficient variant (Δ2) indicated that the *wee* gene cluster controls the synthesis of two polysaccharides: high molecular weight (HMW) and low molecular weight (LMW). LMW polysaccharide of *wee* origin was present in LPS isolated from RAG-1 cells, suggesting a link to the Lipid A-core of LPS molecules. SDS-PAGE analysis indicated that apoemulsan is copurified with LPS polysaccharide, with implications in the emulsification activity of RAG-1 polymer.

Keywords: RAG-1, apoemulsan, *wee* cluster, Wzc, Wzy

Acinetobacter venatianus RAG-1 (ATCC31012) was isolated from seawater in the early 1980s based on its emulsification property [14]. The emulsifying factor produced by RAG-1 is a noncovalently linked complex of proteins and lipoheteropolysaccharide called emulsan [15, 20]. The carbohydrate backbone was proposed to be composed of three aminosugars, D-galactosamine, D-galactosamine uronic acid, and diamino deoxyhexosamine, building a trisaccharide repeating unit [10]. Fatty acids decorate the polysaccharide *via O-* and *N*-acyl binding sites and compromise up to 15% of the emulsan dry weight [1]. The combination of the sugar backbone, which is hydrophilic, with hydrophobic fatty acids gives emulsan its amphipathic character and natural emulsifying features.

Our research is focused on the control of emulsan structure in order to understand the relationships between structure and functional attributes. Previously, we showed that the properties of apoemulsan can be modulated by modifying the fatty acid composition *via* different feeding strategies [9, 19]. Recently, the *wee* gene cluster responsible for apoemulsan synthesis was cloned (Accession No. AJ243431) [12]. According to BLAST searches, putative gene homologs of Wza, Wzb, Wzc, Wzy, and Wzx were identified among the 20 ORFs of the *wee* cluster. These proteins are common in the synthesis of high molecular weight (HMW) polysaccharides in *E. coli via* the Wzy-dependent model [17]. Based on current knowledge of the Wzy-dependent model, we questioned if genetic manipulation might be a new route to modulate apoemulsan properties. It was shown that amino acid substitutions to Wzc [6, 13], or the functional replacement of Wza, Wzb, and Wzc by *E. coli* homologs [7], successfully modified the molecular weight of apoemulsan. For these studies of polymer molecular weight, two deletion mutants were generated, which did not produce HMW apoemulsan: (i) Δwzc where the putative polysaccharide copolymerase (Wzc) was deleted and (ii) apoemulsan-deficient mutant (Δ2) where the promotor region together with six adjacent genes were removed [6]. Surprisingly, both mutants produced polysaccharide that could be recovered from the medium using standard apoemulsan purification protocols. Since this polysaccharide was detected after separation in 8-16% SDS-PAGE at the bottom of the gel, it was termed low molecular weight (LMW) polymer. According to SDS-PAGE gel analysis, LMW polymer was also produced by wild-type RAG-1 cells [6]. These results prompted closer analysis of the polysaccharides produced by *A. venetianus* RAG-1 cells.

For this study the additional deletion mutant (Δwzy) was generated where 503 bp of the putative polymerase gene *wzy* was removed. First, the *wzy* fragment was amplified using primers wzyF (GCA TTC TGA TGT AGT TAA AGA GGT TG) and wzyR (AGT GGC GAT GGA AGT TTA CC) and cloned into the pCR Blunt II TOPO vector. A defined in-frame deletion mutation was constructed by digestion of pCR Blunt II TOPO/*wzy* with BsgI and HpaI, followed by fill-in with Klenow polymerase and re-ligation. The Δ*wzy* gene was excised by digestion with

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SpeI and XhoI, and the cohesive ends were filled in and ligated into the ScaI restriction site of the pSS2141 replacement vector. The truncated *wzy* was introduced into the RAG-1 genome as described previously [6]. Correct genetic rearrangement was confirmed by Southern blot analysis (data not shown).

Complementation plasmid was based on a shuttle vector for *E. coli* and *Acinetobacter* strains pWH1266 [11]. pWH1266 plasmid was cloned by ligation of an *Acinetobacter*-derived plasmid into the PvuII restriction site of pBR322 vector [11]. In order to construct the expression cassette, the primers BlaPF (GGC TGC AGA TAC CAT GGT CTT CCT TTT TCA ATA TT) and BlaPR (GTT GAA GGC TCT CAA GGG CA) were used to amplify a 902-bp fragment of pBR322 vector carrying the bla promoter. The PCR product was digested with PstI and SalI and ligated into the PstI and SalI restriction sites of pWH1266 vector. In order to replace the tet^r gene, the filled-in XbaI-KpnI fragment carrying the kan^r gene under its own promoter was cloned into EcoRV-NruI restriction sites of the modified pWH1266 vector. The resulting plasmid, pWHBK, is kan^r, amp^s, tet^s, and carries the expression cassette where the complemented gene is ligated into the NcoI and PstI restriction sites under control of the bla promoter.

The primers wzc compF (GCC CTG CAG TTA GTC TTC TTT ATT GGC)/wzc compR (GCG CCA TGG GCC AAA ATA CCA ATA CTG AAG) and wzy compF(GAT CTG CAG TTA ATC CCC CCC TGA CCT)/wzy compR (GCG CCA TGG ATA GAT TAG CAT TTA TTG GG) were used to amplify the *wzc* and *wzy* genes, respectively. Forward primers carried PstI, whereas reverse primers carried NcoI restriction sites. Amplified fragments were digested with PstI/NcoI and cloned into complementary sites of pWHBK plasmid. pWHBKwzc and pWHBKwzy vectors were introduced into RAG-1 variants Δwzc and Δwzy, respectively, *via* electroporation. Moreover, the pWHBK vector was electroporated into Δwzc and Δwzy cells for a control study.

The production, purification, and Alcian Blue/SDS-PAGE gel analysis of polymers recovered from culture supernatant were described previously [6]. The preparation of whole-cell lysates and Western blot analysis were performed using protocols described before [7]. The polysaccharides separated by electrophoresis were stained by a SilverXpres Silver Staining Kit (Invitrogen) according the manufacturer's recommendation with minor modification; for fixation, ethanol was used instead of methanol, and for sensitization, periodic acid was applied [16].

In our previous report, we showed that the application of the standard apoemulsan purification protocol from culture medium resulted in copurification of HMW polymer with LMW carbohydrate [6]. Moreover, LMW polymer was recovered from the supernatant of the Δwzc mutant and even from the culture of the apoemulsan-negative mutant (Δ2). The polymer produced by the Δwzy mutant was also purified and analyzed in an Alcian Blue/SDS-PAGE gel (data not shown). HMW apoemulsan was not polymerized, as only LMW polysaccharide was observed in the sample recovered from the Δwzy supernatant. The migration pattern of Δwzy polysaccharide was similar to LMW polysaccharides obtained from RAG-1-Sm, Δwzc, and Δ2 cell cultures.

Since HMW polymer was not present in the samples obtained from Δwzc, Δwzy, and Δ2 mutants, its production was directly dependent on the *wee* gene cluster. In contrast to the LMW polysaccharide LMW production was observed even in the apoemulsan null (Δ2) sample, suggesting a different biosynthesis pathway. In order to investigate the other polysaccharides expressed by RAG-1 and its derivatives, proteinase-K-digested whole-cell lysates were analyzed by silver staining. Two distinguishable polysaccharide bands were observed in the RAG-1-Sm, Δwzc, and Δwzy samples, whereas Δ2 cells generated only one band (Fig. 1A). The application of immunospecific antiserum indicated differences between all analyzed cells (Fig. 1B). The HMW polymer was detected only in the RAG-1 strain. Moreover, immunoreactive LMW polysaccharide was visualized in RAG-1-Sm, Δwzc, and Δwzy cells. RAG-1-Sm and Δwzy cells produced one band of immunospecific LMW materials, Δwzc produced one band as in the RAG-1-Sm and Δwzy variants, and additional immunoreactive extended forms of LMW polysaccharide. The Δ2 mutant did not synthesize immunospecific LMW polysaccharide.

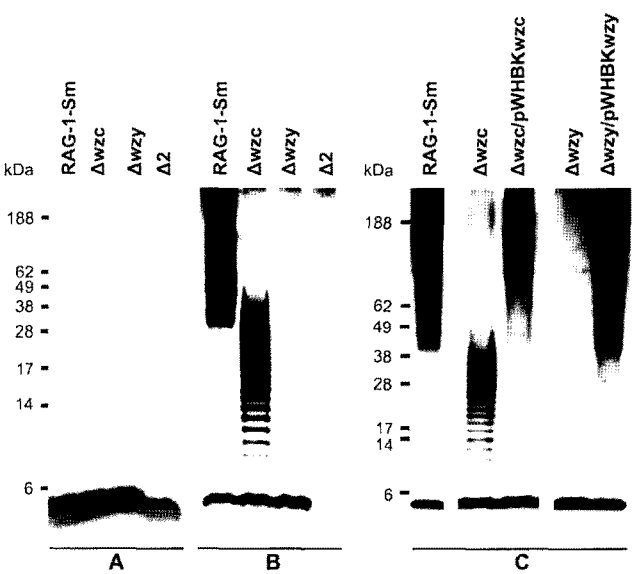

Fig. 1. Analysis of the polysaccharides produced by RAG-1-Sm and its derivatives.

Whole-cell lysates were separated in 16% Tricine gel and visualized with (**A**) silver nitrate or (**B**) processed to the Western blot analysis and probed with anti-apoemulsan specific antiserum. **C**. Complementation assay. The Δwzc and Δwzy mutants were complemented with plasmids expressing *wzc* and *wzy* genes, respectively. Proteinase-K-digested whole-cell lysates were separated in 10–20% Tricine gel and processed to the Western blot analysis using anti-apoemulsan specific antiserum. The mobility of SeeBlue Pre-Stained Standard (Invitrogen) is indicated (kDa).

Moreover, using apoemulsan specific antiserum, Δwzc and Δwzy mutants were analyzed when complemented with plasmid carrying the *wzc* and *wzy* genes, respectively. As shown in Fig. 1C, overexpression of Wzc in the Δwzc variant abolished the formation of the characteristic pattern of extended chains of immunospecific LMW polysaccharide and resulted in the recovery of the production of HMW apoemulsan. The Δwzy derivative also restored the biosynthesis of HMW polymer. Moreover, both complemented strains produced LMW immunoreactive material, in accordance with the general profile of polysaccharides dependent on the *wee* gene cluster in RAG-1 cells. Complementation of Δwzc and Δwzy variants with plasmid pWHBK did not result in the recovery of the ability to synthesize HMW polymer (data not shown). These result confirmed two important outcomes: (i) the loss of HMW apoemulsan was directly correlated with deletion of particular genes and no polar effect was observed, and (ii) the antibody was specific to polysaccharide whose production was dependent on the *wee* gene cluster.

The existence of LMW polysaccharide of *wee* cluster origin and the generation of longer chains by the Δwzc mutant resembles the production of K_{LPS} in *E. coli*. The *E. coli* K30 capsule is expressed at the cell surface in two forms:

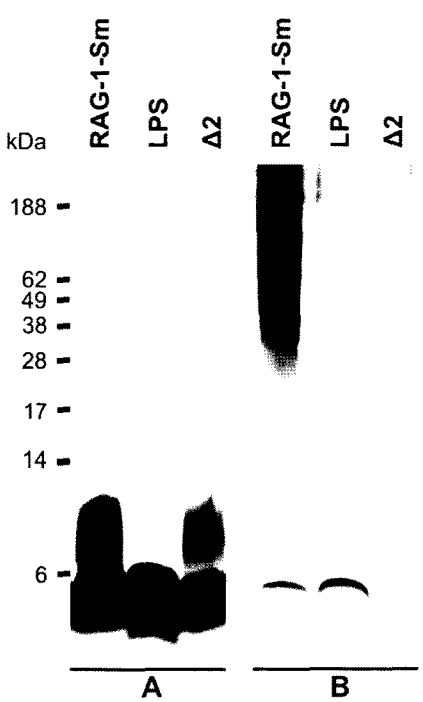

Fig. 2. SDS-PAGE analysis of purified LPS fraction and polysaccharides recovered from RAG-1-Sm and Δ2 culture media.

After separation in 16% Tricine, gel samples were visualized with (**A**) silver nitrate or (**B**) processed to the Western blot analysis and probed with anti-apoemulsan specific antiserum. The mobility of SeeBlue Pre-Stained Standard (Invitrogen) is indicated (kDa).

(i) one is HMW polymer, evident in electron micrographs as higher-order structures on the cell surface; and (ii) another is LMW polymer linked to an LPS Lipid A-core termed K_{LPS} [17]. Deletion of K30 *wzc* or *wza* abolished the formation of HMW polysaccharide and increased the extent of polymerization of K_{LPS} [8, 18]. In order to evaluate whether the immunoreactive LMW carbohydrate may be the counterpart of *E. coli* K30 K_{LPS}, we recovered the LPS from RAG-1 cells using a method specific for LPS extraction [2]. As shown in Fig. 2B, the HMW apoemulsan polymer was immunodetected only in the polysaccharide sample obtained from RAG-1-Sm cell supernatant. Moreover, the *wee* cluster-dependent LMW polysaccharide was immunodetected in LPS purified from RAG-1 cells and polymers obtained from the supernatant of RAG-1-Sm cells. Immunoreactive LMW polysaccharide was not present in the polysaccharide purified from the Δ2 supernatant. The silver staining indicated a similar migration pattern of purified LPS and LMW polysaccharide recovered from RAG-1-Sm and Δ2 supernatants (Fig. 2A). Since polysaccharides show a tendency to form aggregates, the additional weaker silver-stained signal above the strong signal for LMW polysaccharide in the RAG-1-Sm and Δ2 samples may have resulted from aggregates.

Immunodetection of LMW *wee*-dependent polysaccharide in LPS fractions purified from the cells suggested that this LMW carbohydrate might be a counterpart to *E. coli* K_{LPS}. However, the LMW *wee*-dependent polymer has not been purified, and since we do not know the chemical composition, we cannot be sure whether it is composed of the same sugar repeat unit as HMW apoemulsan. Analysis by silver-stained gels of whole-cell lysates indicated the absence of one polysaccharide band in the Δ2 sample, which migrated at the same level as immunodetected LMW polysaccharide in the other samples (RAG-1-Sm, Δwzc, Δwzy). Since we purified the anti-apoemulsan antiserum by adsorption on the Δ2 mutant, the antibody recognizing this particular polysaccharide band could stay in the solution. The *wee* gene cluster is directly responsible for synthesis of this LMW immunoreactive polysaccharide; however, we cannot preclude that some glycosytransferases present in the *wee* cluster are involved in the synthesis of sugars needed for assembly of different repeat units.

It is interesting that the complementation study of the *wzc* gene resulted in a more narrow molecular weight distribution of HMW apoemulsan. In the *E. coli* K30 model, the Wzc protein was shown to form a tetramer [4]. The Wzc oligomer is complexed with the Wza octamer [5]. Recently, it was proposed that conformational changes of the Wzc complex may serve as a focal point for further protein-protein interactions in a complex controlling biosynthesis and export of HMW polysaccharides [5]. Complementation studies are known to disturb the natural stoichiometry of the proteins. Since the Δwzc mutant complemented with

plasmid expressing *wzc* resulted in apoemulsan production with a modified molecular weight distribution, this may indicate that stoichiometry is important in the regulation of the biosynthesis and export of HMW carbohydrates.

Genetic analysis of apoemulsan production by RAG-1 indicated two important outcomes. First, the polymerization of apoemulsan was conducted in a similar fashion to the polysaccharide biosynthesis model described for several *E. coli* strains [17]. The use of antibodies as molecular probes revealed the presence of both HMW and LMW *wee*-dependent polysaccharides, where the LMW formed associated with LPS fractions extracted from RAG-1 cells and may be the counterpart of K_{LPS}. A second outcome is connected with the analysis of polymers purified from the supernatant of deletion mutants and especially the apoemulsan-negative mutant. The results indicated that methods used for purification of apoemulsan resulted in the copurification of two different polymers: one dependent and another independent of the *wee* gene cluster. Western blot analysis of purified polymers indicated the presence of immunoreactive LMW polysaccharide in the RAG-1-Sm sample, but not in the Δ2 material. However, LMW polysaccharide was stained in the Δ2 samples using Alcian Blue and silver nitrate. This result indicated that the observed LMW polysaccharide was a mixture of at least two different components. The polysaccharide recovered from the Δ2 supernatant migrated on the 16% Tricine gel with the same pattern as purified LPS. These data suggest that standard purification methods used for apoemulsan polymer result in the copurification with LPS molecules. The presence of LPS in the culture medium of *A. venetianus* RAG-1 cells is not unexpected. Brade and Galanos [2] showed that, during growth of *A. calcoaceticus* NCTC 10305, only 40% of the synthesized lipopolysaccharide remained cell-bound, while a larger part was released into the culture medium. The same LPS molecules, based on chemical analysis, were found in the medium as on the surface of the cells.

The LPS molecule is composed of a lipid moiety called Lipid A, a core of about 10 monosaccharides, and a smooth-type LPS, another region named O-chain built of repetitive units of polysaccharide [3]. The lipid moiety is a common general architecture determined as a disaccharide substituted with fatty acids [3]. Apoemulsan is described as a polysaccharide composed of repeat units of aminosugars decorated with fatty acids [20]. Since the general structure of Lipid A and apoemulsan are similar and we found the LPS molecules to be copurified with apoemulsan, a more precise analysis of the apoemulsan preparation is needed.

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