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Involvement of a Protein Tyrosine Kinase in Production of the Polymeric Bioemulsifier Emulsan from the Oil-Degrading Strain *Acinetobacter lwoffii* RAG-1

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The genes associated with the biosynthesis of the polymeric bioemulsifier emulsan, produced by the oil-degrading *Acinetobacter lwoffii* RAG-1 are clustered within a 27-kbp region termed the *wee* cluster. This report demonstrates the involvement of two genes of the *wee* cluster of RAG-1, *wzb* and *wzc*, in emulsan biosynthesis. The two gene products, Wzc and Wzb were overexpressed and purified. Wzc exhibited ATP-dependent autophosphorylating protein tyrosine kinase activity. Wzb was found to be a protein tyrosine phosphatase capable of dephosphorylating the phosphorylated Wzc. Using the synthetic substrate *p*-nitrophenyl phosphate (PNPP) Wzb exhibited a V_{max} of 12 μmol of PNPP min^{-1} mg^{-1} and a K_m of 8 mM PNPP at 30°C. The emulsifying activity of mutants lacking either *wzb* or *wzc* was 16 and 15% of RAG-1 activity, respectively, suggesting a role for the two enzymes in emulsan production. Phosphorylation of Wzc was found to occur within a cluster of five tyrosine residues at the C terminus. Colonies from a mutant in which these five tyrosine residues were replaced by five phenylalanine residues along with those of a second mutant, which also lacked Wzb, exhibited a highly viscous colony consistency. Emulsan activity of these mutants was 25 and 24% of that of RAG-1, respectively. Neither of these mutants contained cell-associated emulsan. However, they did produce an extracellular high-molecular-mass galactosamine-containing polysaccharide. A model is proposed in which subunit polymerization, translocation and release of emulsan are all associated and coregulated by tyrosine phosphorylation.

The hydrocarbon-degrading organism *Acinetobacter lwoffii* RAG-1 produces an extracellular, polymeric, galactosamine-containing lipoheteropolysaccharide bioemulsifier (molecular mass, 10^3 kDa), termed emulsan (31, 43). Because of its properties as an emulsion stabilizer, emulsan has been studied extensively and its chemical composition, physical properties, physiology, and fermentation along with its industrial applications have been reviewed (5, 16, 17). Briefly, emulsan is composed of a linear repeating trisaccharide subunit consisting of *N*-acyl-D-galactosamine, *N*-acyl-L-galactosamine uronic acid and 2,4-diamino-6-deoxy-D-glucosamine (2, 16, 43). The amphipathic properties of emulsan are due in part to the presence of about 15% fatty acids covalently bound to the water-soluble biopolymer in both ester and amide linkages (2). During growth on minimal medium, the biopolymer accumulates on the cell surface of exponential-phase RAG-1 cells as a minicapsule and is released into the medium as a protein-polysaccharide complex as the cells approach stationary phase (11, 29). The protein-free polysaccharide termed apoemulsan is less effective than emulsan in forming emulsions with hydrophobic substrates, although it does stabilize preformed emulsions (35, 42). An exocellular esterase of RAG-1, cloned and sequenced in *Escherichia coli*, was found to enhance the emulsification of very hydrophobic substrates by apoemulsan in a fashion which did not depend on catalytic activity (H. Bach and D. L. Gutnick, 2002; submitted).

Recently, the genes encoding the biosynthetic pathway of apoemulsan were sequenced and localized to a single gene cluster, the *wee* cluster, consisting of 20 open reading frames (25). Three genes of this cluster, *wza*, *wzb*, and *wzc*, were predicted from sequence homology to be involved in the assembly of emulsan on the cell surface (25). Multimers of the *E. coli* Wza homologue have been proposed to form an outer membrane pore through which the capsular antigen is translocated (8). Wzc homologues, members of the MPA1 family, were shown to autophosphorylate protein tyrosine kinases localized to the inner membrane (14, 20, 27, 38). These proteins contain an ATP-binding motif, which is necessary for tyrosine phosphorylation (6, 23, 26, 41). Wzb homologues were demonstrated to be protein tyrosine phosphatases (3, 15, 38, 39), capable of dephosphorylating Wzc (38, 39).

In this work we show that Wzb and Wzc of RAG-1 constitute a protein tyrosine phosphatase and an autophosphorylating protein tyrosine kinase, respectively. Based on the analysis of *wzb* and *wzc* mutations, we propose a model for the involvement of Wzb and Wzc in emulsan biosynthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial stains and plasmids used in this study are listed in Table 1.

Culture media and growth conditions. *A. lwoffii* strains were cultivated in ethanol-minimal salts medium (34) supplemented with Hutner's Metals 44 (4) at 30°C. *E. coli* strains were grown on Luria-Bertani broth at 37°C. The antibiotics ampicillin, kanamycin, and tetracycline were added to the media at concentrations of 100, 75, and 15 μg/ml, respectively.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
A. lwoffii		
RAG-1	Wild type, emulsan producer	30
LN201	Emulsan-deficient mutant of RAG-1, *wzc*::miniTn*10*	25
LN401	LN201 carrying plasmid pDLG1	This work
LN501	Emulsan-deficient mutant of RAG-1, Δ*wzb*	This work
LN502	Emulsan-deficient mutant of RAG-1, Δ*wzc*	This work
LN503	Mutant of RAG-1, highly viscous colony morphology, $wzc_{(Y712F,\ Y714F,\ Y716F,\ Y718F,\ Y720F)}$	This work
LN504	Mutant of RAG-1, highly viscous colony morphology, Δ*wzb* $wzc_{(Y712F,\ Y714F,\ Y716F,\ Y718F,\ Y720F)}$	This work
E. coli		
XL-1 Blue	*supE hsd*Δ5 *thi* (*lac-proAB*) {F′ *tra*D36 *pro*AB$^+$ *lacI*^{q} *lacZ*ΔM15}	Stratagene
BL21 (DE3)	F$^-$ *ompT hsdB* (r^-_B m^-_B) *dcm gal* (DE3)	36
K10	K10 capsule producer	I. Ørskov and F. Ørskov
Plasmids		
pWH1274	Shuttle vector for *E. coli* and *Acinetobacter* strains, Tet^r Amp^r	19
pDLG-1	pWH1274 derivative constitutively expressing Wzc	This work
pET-14b	T_7 expression vector, Amp^r	Novagen
pET-*wzb*	pET-14b derivative expressing Wzb with an N-terminal His tag, Amp^r	This work
pET-*wzc*	pET-14b derivative expressing Wzc with an N-terminal His tag, Amp^r	This work
pJP5603	Suicide vector, Kan^r *oriR6K mobRP4*	28
pJPdel-*wzb*	pJP5603 carrying a fragment containing the nucleotide sequence of the N terminus (Wzb_{1-11}) and C terminus ($Wzb_{124-142}$) of Wzb	This work
pJPdel-*wzc*	pJP5603 carrying a fragment containing the nucleotide sequence of the N terminus (Wzc_{1-4}) and C terminus ($Wzc_{721-726}$) of Wzc	This work
pJPdel-5Y	pJP5603 carrying a fragment with nucleotide substitutions resulting in amino acid changes Y712F, Y714F, Y716F, Y718F, and Y720F of Wzc	This work

General DNA manipulations. A DNA High Pure plasmid isolation kit (Roche) was used for small-scale plasmid isolations. The High Pure PCR product purification kit (Roche) was used for the purification of PCR products and DNA from agarose gel. All restriction enzymes (Fermentas), T7 DNA polymerase (Takara), and alkaline phosphatase and T4 DNA ligase (Roche) were used as recommended by the manufacturers. Transformation of *E. coli* and *A. lwoffii* strains was performed as described previously (33). Southern hybridization was performed using the DIG-High prime DNA labeling kit (Roche). Sequencing was performed using an ABI 377 DNA sequencing apparatus (Perkin-Elmer).

Construction of pDLG1, the *wzc* expression plasmid. One PCR was performed to amplify the promoter region of the *bla* gene of pWH1274 using primers WHP1 and WHP2, and one was performed to amplify the *wzc* gene of RAG-1 using primers WHC1 and WHC2. The sequences of the primers are listed in Table 2. The fragment containing the *bla* promoter region was cut with *Cla*I and *Nco*I, and the fragment containing *wzc* was cut with *Bsp*HI and *Pst*I. Both fragments together were ligated into a *Cla*I/*Pst*I-linearized pWH1274 plasmid. In the resulting pDLG1 plasmid, *wzc* is under the control of the *bla* promoter.

Construction of the *wzc* and *wzb* overexpression plasmids. The gene *wzb* was amplified by PCR using primers ETB1 and ETB2, and *wzc* was amplified using primers ETC1 and ETC2. The sequences of the primers are listed in Table 2. Both PCR products were digested with *Nde*I and *Bam*HI and cloned separately into the *Nde*I/*Bam*HI-linearized plasmid pET14b, which resulted in the plasmids pET14b-*wzb* and pET14b-*wzc*, respectively.

Constructing chromosomal deletions and point mutations. Two plasmids were constructed to create internal deletions of both *wzb* and *wzc*. The sequences of the primers are listed in Table 2. Plasmid pJPdel-*wzb* was constructed by PCR amplification of two fragments using primers DLB1 and DLB2 (the region upstream of *wzb*) and primers DLB3 and DLB4 (the region downstream of *wzb*). The first fragment was cut with *Eco*RI and *Not*I, and the second fragment was cut with *Bam*HI and *Not*I, and both were ligated into an *Eco*RI/*Bam*HI-linearized pJP5603 plasmid. The resulting plasmid contained the sequence coding for the first 11 amino acids and the last 19 amino acids of Wzb lacking the internal portion of the gene. Plasmid pJPdel-*wzc* was constructed by PCR amplification of two fragments using primers DLC1 and DLC2 (the region upstream of *wzc*) and primers DLC3 and DLC4 (the region downstream of *wzc*). The first fragment was cut with *Xba*I and *Not*I, and the second fragment was cut with *Eco*RI and *Not*I; both were ligated into an *Xba*I/*Eco*RI-linearized pJP5603 plasmid. The resulting plasmid contained the sequence coding for the first 4 amino acids and the last 6 amino acids of *wzc* lacking the internal portion of the gene.

Defined point mutations in *wzc* were constructed using overlap-extension PCR as described previously (18). Two fragments were amplified using primers FPY1 and FPY2 (the region upstream of the tyrosine cluster) and primers FPY3 and DLC4 (the region downstream of the tyrosine cluster). Another amplification was done using primers FPY1 and DLC4 and both PCR fragments as template. In the resulting fragment, five tyrosine codons at the C terminus of *wzc* were converted to phenylalanine codons. This fragment was cut with *Xba*I and *Eco*RI and ligated into an *Xba*I/*Eco*RI-linearized pJP5603 plasmid, which resulted in plasmid pJPdel-5Y.

These plasmids were used to transform RAG-1. Kanamycin-resistant transformants were isolated and subsequently screened for the subsequent loss of kanamycin resistance after growth without selectable marker. Defined in-frame deletions and point mutations were confirmed using appropriate PCRs, Southern analysis, and DNA sequencing (data not shown).

Purification of Wzb and Wzc. *E. coli* BL21(pET14b-*wzb*) cells were grown in Luria-Bertani medium containing ampicillin at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6. Induction was started by adding 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) followed by incubation for 3 h. His-tagged Wzb was purified according to the "pET System Manual" of Novagen. The protein was eluted with 250 mM imidazole, dialysed 12 h against TBS (150 mM NaCl, 20 mM Tris [pH 7.5]), and stored in 50% (vol/vol) glycerol at −20°C.

Wzc expression by *E. coli* BL21(pET14b-*wzc*) was done as described for CelG of *C. cellulolyticum* (10). His-tagged Wzc was purified similarly to Wzb except that 0.1% (vol/vol) Triton X-100 was added to the lysate after sonication and elution was done with 500 mM imidazole. Wzc was purified freshly before every experiment.

Western immunoblotting. Bacterial cell lysates from overnight *A. lwoffii* cells grown on ethanol-minimal salts medium were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (22) prior to transfer onto nitrocellulose (37). The nitrocellulose filters were incubated with either antiphosphotyrosine antibodies (PT-66; Sigma) at a dilution of 1:2,000 or polyclonal mouse anti-Wzc of *A. lwoffii* RAG-1 antiserum (1:200). Mouse anti-Wzc antiserum was obtained following immunization of BALB/c female mice with purified His-tagged Wzc.

TABLE 2. Primers used in this study

Primer no.	Name[a]	Sequence[b]	Primer feature(s)[c]
1	WHC1 (+)	5′-GGGATCC**TCATGA**GCCAAAATACCAATACTG-3′	Wzc, *Bsp*HI, start codon
2	WHC2 (−)	5′-GGGG**CTGCAG**TTAGTCTTCTTTATTGGC-3′	Wzc, *Pst*I, stop codon
3	WHP1 (−)	5′-GGGGC**CATGG**TCTTCCTTTTTCAATATTATTGAAGC-3′	*bla* promoter, *Nco*I, start codon
4	WHP2 (+)	5′-TAACTGTGATAAACTACCG-3′	*bla* promoter, upstream *Cla*I
5	ETC1 (+)	5′-GGGG**CATATG**AGCCAAAATACCAATACTG-3′	Wzc, *Nde*I, start codon
6	ETC2 (−)	5′-CCCC**GGATCC**TTAGTCTTCTTTATTGGC-3′	Wzc, *Bam*HI, stop codon
7	ETB1 (+)	5′-GGGG**CATATG**AATATTAAAAACGTTTTAGTTG-3′	Wzb, *Nde*I, start codon
8	ETB2 (−)	5′-CC**GGATCC**TTAAATATAGTTTTTCCAATCTG-3′	Wzb, *Bam*HI, stop codon
9	DLB1 (+)	5′-CCG**GAATTC**TTTCAGCTAGAATTAGCGC-3′	$Wzb_{1–11}$, *Eco*RI
10	DLB2 (−)	5′-ATAAGAAT**GCGGCCGC**CACACAAACAACTAAAACG-3′	$Wzb_{1–11}$, *Not*I
11	DLB3 (+)	5′-ATAAGAAT**GCGGCCGC**TTTTGATCAGACCTGTCTGC-3′	$Wzb_{124–142}$, *Not*I
12	DLB4 (−)	5′-CGC**GGATCC**AAAGTTGGAGGTATTGACGC-3′	$Wzb_{124–142}$, *Bam*HI
13	DLC1 (+)	5′-CTAG**TCTAGA**TCCTCTCTCAGCTAGTGCG-3′	$Wzc_{1–4}$, *Xba*I
14	DLC2 (−)	5′-AAATAT**GCGGCCGC**ATTTTGGCTCATAATCTTAAATC-3′	$Wzc_{1–4}$, *Not*I
15	DLC3 (+)	5′-AAATAT**GCGGCCGC**TAAAGCCAATAAAGAAGAC-3′	$Wzc_{721–726}$, *Not*I
16	DLC4 (−)	5′-CCG**GAATTC**AATTGGCTCGCC-3′	$Wzc_{721–726}$, *Eco*RI
17	FPY1 (+)	5′-CTAG**TCTAGA**TCCTGTACAAGAAACTCG-3′	*Xba*I
18	FPY2 (−)	5′-AGGCAaAAGCAaAGTTAaAGCTAaAACCAGCGCCAGCAGTACG-3′	$Wzc_{(Y712F, Y714F, Y716F, Y718F, Y720F)}$
19	FPY3 (+)	5′-TAGCTtTAACTtTGCTTtTGCCTtTAAAGCCAATAAAGAAGAC-3′	$Wzc_{(Y712F, Y714F, Y716F, Y718F, Y720F)}$

[a] + and −, forward and reverse primers, respectively.
[b] Underlined sequences are start or stop codons. Sequences in boldface type are restriction sites. Lowercase letters are nucleotides altered from those in the chromosome to change codons coding for tyrosine into codons coding for phenylalanine.
[c] Primers used for amplification of a fragment encoding a particular polypeptide or for generating defined point mutations are described. Restriction sites and start or stop codons are listed.

The filter was then incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibodies (Jackson Immunoresearch) and reacted with ECL reagent (Amersham). Low-range prestained SDS-polyacrylamide gel electrophoresis (PAGE) standards (Bio-Rad) were used as molecular weight markers.

In vitro phosphorylation assay of Wzc. Monitoring autophosphorylation of Wzc using [γ-^{32}P]ATP was performed as described previously (20). Aliquots were taken at different time points and used for analysis by SDS-PAGE. The gel was dried using a model 583 gel dryer (Bio-Rad), and a film was exposed to the dried gel for the appropriate time and developed.

Phosphoamino acid analysis. Purified Wzc was labeled in vitro with [γ-^{32}P]ATP or [α-^{32}P]ATP as described above. Labeled Wzc was hydrolyzed and analyzed by ascending thin-layer chromatography (TLC) (TLC cellulose; Merck Inc.) as described previously (9, 20). Phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) were used as markers and visualized as described previously (9).

Phosphatase activity of Wzb. Acid phosphatase activity was monitored at 30°C by using the continuous method based on the cleavage of *p*-nitrophenyl phosphate (PNPP) previously described (38).

Dephosphorylation of Wzc was monitored by Western hybridization. Wzc was incubated with and without Wzb as described (38). Aliquots were taken at the appropriate time and analyzed by Western hybridization using antiphosphotyrosine antibodies.

Standard assay for emulsan activity. Cell-free emulsifying activity was determined by the standard assay as described previously (31). One unit of emulsan activity per ml was defined as that amount which increases turbidity by 100 Klett units.

Galactosamine content. Galactosamine content was determined using indole after deamination with nitrous acid (1). Cell-associated galactosamine was determined on a concentrated cell suspension. The cells were washed once in cold distilled water and resuspended (OD_{600} = 10).

Separation of polysaccharides on SDS-polyacrylamide gel. Samples of the supernatant and the resuspended cells (OD_{600} = 10) of each strain were prepared as described previously (21) and separated by SDS–6% PAGE (22). Polysaccharides were stained with Alcian blue (Sigma) as described previously (21). Broad-range prestained SDS-PAGE standards (Bio-Rad) were used as molecular weight markers.

Composition of high-molecular-mass polymer from LN503 and *A. lwoffii* RAG-1. Emulsan and the high-molecular-mass polysaccharide produced by LN503 were isolated, purified, and deproteinized as described previously (43). Following acid hydrolysis the hydrolysates were analyzed by TLC (43).

RESULTS

Wzc of *A. lwoffii* RAG-1 is an autophosphorylating protein tyrosine kinase. The putative translation sequence of *wzc* of RAG-1 exhibited high similarity to several autophosphorylating protein tyrosine kinases involved in polysaccharide biosynthesis in other bacteria (25). In order to examine whether Wzc of RAG-1 is in fact a tyrosine-phosphorylated protein, lysates of the parent, RAG-1 (Fig. 1, lane 1); emulsan-deficient mutant LN201, containing the miniTn*10*Km transposon inserted into the *wzc* gene (Fig. 1, lane 2); and LN401, LN201 carrying plasmid pDLG1 (Fig. 1, lane 3), were subjected to Western immunoblot analysis using antiphosphotyrosine antibodies. The pDLG1 plasmid is a shuttle vector, which constitutively expresses the *wzc* gene of RAG-1 from the *bla* promoter (Materials and Methods). The strains are listed in Table 1. The antibodies cross-reacted with a protein from the lysates of wild-type RAG-1 and LN401. However, no cross-reacting protein was detected in the mutant lysate in the absence of the plasmid. The molecular mass of the protein (approximately 82 kDa) was in close agreement with the value calculated from the amino acid composition of Wzc (25). The colonial morphology of LN201 was translucent (Tlu), in contrast to the opaque morphology of wild-type, emulsan-producing RAG-1 (25). In addition, ethanol-grown cultures of RAG-1 exhibited extracellular emulsan activities of 220 U/ml, while mutant LN201 showed only 18 U/ml (25). In sharp contrast, strain LN401 showed an emulsifying activity of 215 U/ml and opaque colonial morphology characteristic of RAG-1. These results taken together strongly suggest that Wzc is a tyrosine phosphorylated protein, which is required for emulsan production.

To demonstrate that Wzc is an autophosphorylating protein kinase, purified His-tagged Wzc was incubated with [γ-^{32}P]ATP.

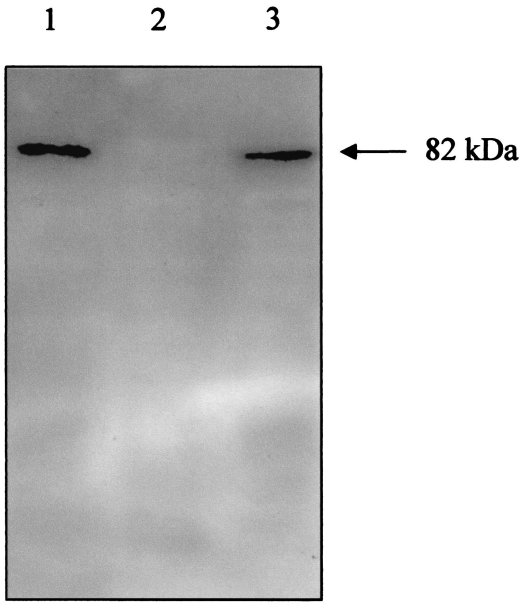

FIG. 1. Presence of phosphorylated Wzc in various *A. lwoffii* RAG-1 mutants. Lysates of the RAG-1 mutants were immunoblotted using antiphosphotyrosine antibodies (PT66) as described in Materials and Methods. Lane 1, wild-type RAG-1; lane 2, LN201; lane 3, LN401.

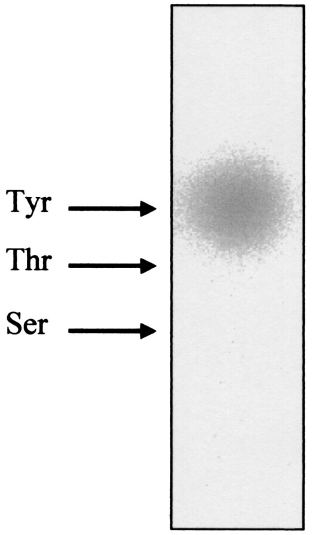

FIG. 3. Phosphoamino acid analysis of Wzc. Purified His-tagged Wzc was used in a protein kinase assay with [γ-^{32}P]ATP as a phosphate donor. The protein was hydrolyzed and subsequently run on a TLC cellulose plate as described in Materials and Methods.

At various times, samples were removed and subjected to SDS-PAGE, and the gel was subjected to autoradiography. It can be seen from the autoradiogram presented in Fig. 2 that the extent of Wzc phosphorylation increased with time over a period of 4 h. In order to identify which amino acid was phosphorylated, ^{32}P-labeled Wzc was subjected to acid hydrolysis, and the hydrolysate was analyzed by TLC as described in Materials and Methods. Autoradiography of the chromatogram revealed the presence of a single phosphoamino acid, phosphotyrosine (Fig. 3). This experiment was repeated with [α-^{32}P]ATP as a phosphate donor to ensure that the presence of phosphotyrosine was due to kinase activity. In this case no phosphotyrosine or any other phosphoamino acids were detected (data not shown). The results indicate that Wzc is an autophosphorylating protein tyrosine kinase.

Wzb of *A. lwoffii* RAG-1 is a protein tyrosine phosphatase. Purified His-tagged Wzb was found to cleave the synthetic substrate PNPP with a V_{max} of 12 μmol PNPP min^{-1} mg^{-1} and a K_m of 8 mM PNPP at 30°C. To examine the dephosphorylation of Wzc by Wzb, purified His-tagged Wzc was incubated with purified His-tagged Wzc at 30°C, and at various times samples were removed and analyzed by Western immunoblot analysis using antiphosphotyrosine antibodies (Fig. 4). It can be seen that in the presence of Wzb, Wzc was dephosphorylated over time, with complete dephosphorylation occurring within the first 12 h of incubation. In the absence of Wzb or in the presence of boiled Wzb (not shown) there was no dephosphorylation of the kinase.

Mutants of *A. lwoffii* RAG-1 with deletions of Wzb and Wzc. To further characterize the requirements for Wzb and Wzc in emulsan production a series of mutants were introduced into RAG-1 and characterized for extracellular emulsifying activity and biopolymer production. Deletion mutants of both *wzb* (*A. lwoffii* LN501) and *wzc* (*A lwoffii* LN502) were generated using the suicide vectors pJPdel-*wzb* and pJPdel-*wzc*, respectively, as described in Materials and Methods. The strains and plasmids are listed in Table 1. Colonies of mutants LN501 or LN502 exhibited Tlu colony morphology characteristic of emulsan-defective mutants (not shown). In addition, Western analysis using either anti-Wzc antibody or antiphosphotyrosine antibody (Fig. 5) revealed that strain LN502 was deleted from the 82-kDa Wzc protein. This protein was still present in LN501, showing that the deletion of Wzb did not affect downstream expression of Wzc.

Mutants in *wzc* of *A. lwoffii* RAG-1 with deletions of the phosphorylation site(s). Sequence analysis of *wzc* genes from a number of organisms has suggested that a tyrosine cluster

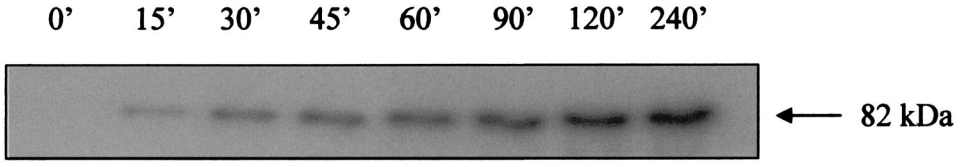

FIG. 2. Autophosphorylation of Wzc. Purified His-tagged Wzc was incubated with [γ-^{32}P]ATP as a phosphate donor as described in Materials and Methods. The reaction was stopped at 0, 15, 30, 45, 60, 90, 120, and 240 min by adding sample buffer. An autoradiograph of these samples run on an SDS-polyacrylamide gel was developed.

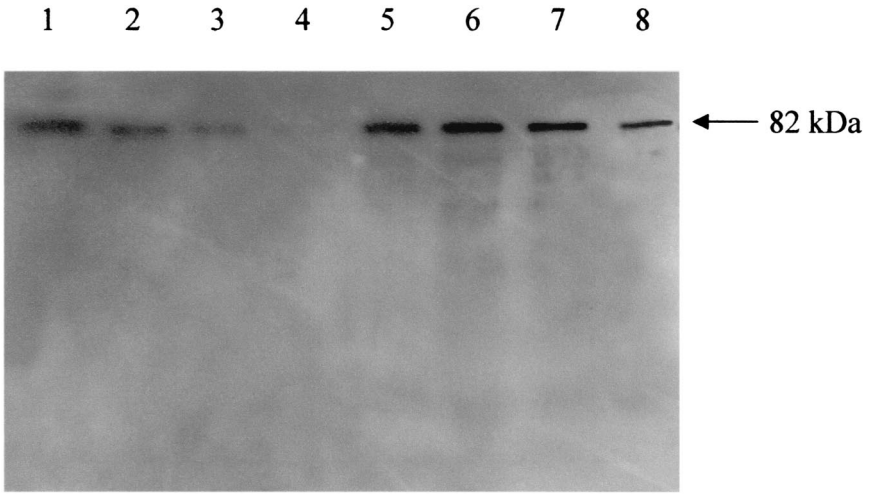

FIG. 4. In vitro dephosphorylation of Wzc by Wzb. Purified His-tagged Wzc was incubated at 30°C with purified Wzb as described in Materials and Methods. The reaction was stopped at 0, 30, and 60 min and 12 h, and aliquots were analyzed by Western immunoblotting using antiphosphotyrosine antibodies (PT66). As a control Wzc was incubated without Wzb. The following lanes were incubated with Wzb for the indicated times: lane 1, 0 min; lane 2, 30 min; lane 3, 60 min; lane 4, 12 h. The following lanes were incubated without Wzb for the indicated times: lane 5, 0 min; lane 6, 30 min; lane 7, 60 min; lane 8, 12 h.

located at the C terminus of these Wzc homologues is a conserved site(s) for tyrosine phosphorylation (23). Wzc of RAG-1 was found to contain a tyrosine cluster which could be aligned with similar clusters from several Wzc homologues (not shown). Accordingly, employing the approach described in Materials and Methods, a mutant strain was constructed in which these five tyrosine residues at the C terminus of Wzc were replaced by five phenylalanine residues. This strain, *A. lwoffii* LN503, was created using plasmid pJPdel-5Y. In addition, *A. lwoffii* LN504 was deleted for *wzb* and lacked the tyrosine cluster. The Western immunoblot analysis presented in Fig. 5 clearly shows that mutants LN503 and LN504, both of which lack the tyrosine cluster, still produced the 82-kDa Wzc protein. However, this protein was not phosphorylated. Finally, unlike strains LN501 and LN502 in which the colonial morphology was Tlu, colonies of LN503 and LN504 were opaque and exhibited a highly viscous, gum-like consistency.

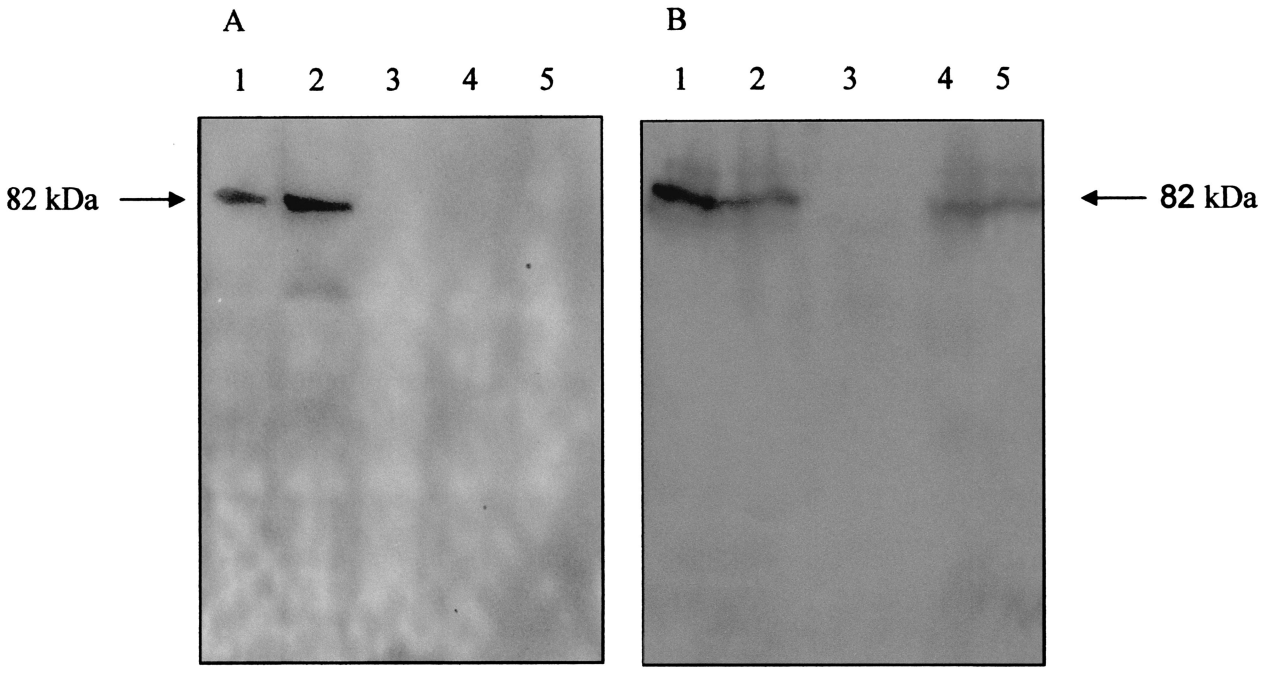

FIG. 5. Presence of the (phosphorylated) Wzc protein in defined *A. lwoffii* RAG-1 mutants. Lysates of the RAG-1 mutants were immunoblotted using antiphosphotyrosine antibodies (A) and anti-Wzc (B) as described in Materials and Methods. Lane 1, wild-type RAG-1; lane 2, LN501; lane 3, LN502; lane 4, LN503; lane 5, LN504.

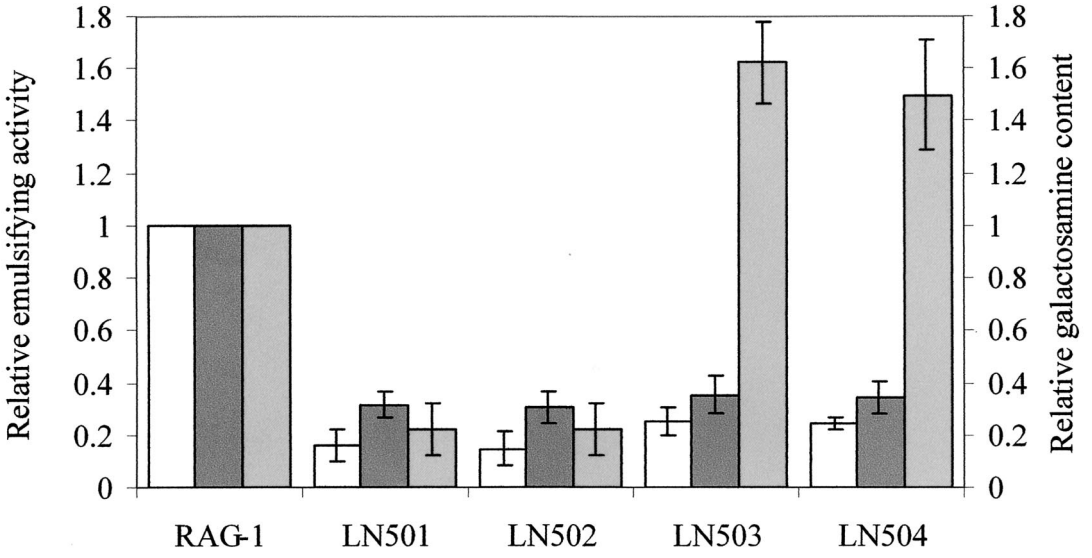

FIG. 6. Emulsifying activities and galactosamine content of defined mutants of *A. lwoffii* RAG-1 compared to the wild type. Relative emulsifying activity and relative galactosamine content were determined as described in Materials and Methods. The emulsifying activity of RAG-1 is 210 U/ml, the cell-associated galactosamine content of RAG-1 is 106 μg/OD_{600}, and the galactosamine content in the supernatant of RAG-1 is 1,170 μg/ml. Relative emulsifying activity (white bars), relative cell-associated galactosamine content (dark-grey bars), and relative galactosamine content in the supernatant (light-grey bars). The results are the averages ± standard deviations (error bars) of at least three different experiments.

Emulsifying activity and polymer production in mutants of *A. lwoffii* RAG-1. The Tlu morphology of deletion mutants LN501 and LN502 suggested that these mutants were defective in emulsan production. As shown in Fig. 6 these two strains along with LN503 and LN504, which make nonphosphorylating Wzc, were all defective in emulsifying activity. The emulsifying activity of these mutants was between 15% (22 U/ml) and 25% (33 U/ml) of the wild-type activity (210 U/ml).

Previous results demonstrated that the major source of cellular galactosamine in RAG-1 is in the apoemulsan polymer (43). As expected, the galactosamine content of the cells and that of the supernatant in mutants LN501 and LN502 were correlated with the low emulsan activity (Fig. 6). However, mutants LN503 and LN504 exhibited much higher levels (62 and 50%, respectively) of galactosamine in the supernatant than that of parental strain RAG-1 (Fig. 6). Interestingly, the galactosamine content of the cells of mutants LN503 and LN504 was much lower than in cells of the parent.

The high galactosamine content in the broth of mutants LN503 and LN504 was also consistent with the finding that a high-molecular-mass polysaccharide was present in these mutants which was not present in any of the other strains examined (Fig. 7).

The high-molecular-mass polysaccharide produced by mutant LN503 was isolated and purified along with emulsan itself as described previously (43). After deproteinization by hot phenol, the polymers were extensively dialyzed and subjected to strong-acid hydrolysis (43). The sugar composition of the hydrolysates was analyzed by TLC (43). TLC chromatograms of the two preparations were indistinguishable when stained either for reducing sugar or with ninhydrin for amino sugars (not shown).

DISCUSSION

In a previous report (25) it was demonstrated that the genes encoding the enzymes involved in the biosynthesis of emulsan are localized within the 27-kbp *wee* cluster. In this report we demonstrate that Wzc and Wzb are a protein tyrosine kinase and protein tyrosine phosphatase, respectively. Deletions in

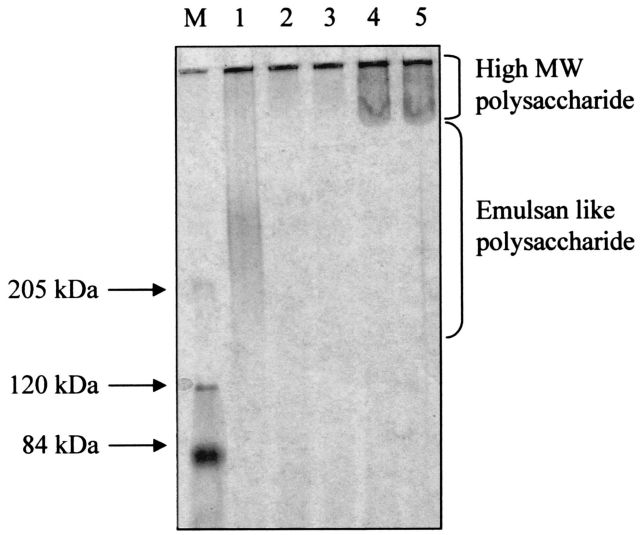

FIG. 7. Size distribution of the polysaccharides in the supernatant of defined mutants of *A. lwoffii* RAG-1 compared to the wild type. Samples of the supernatant were run on an SDS-polyacrylamide gel and stained with Alcian blue as described in Materials and Methods. Lane M, broad-range protein marker. Lane 1, wild-type RAG-1; lane 2, LN501; lane 3, LN502; lane 4, LN503; lane 5, LN504.

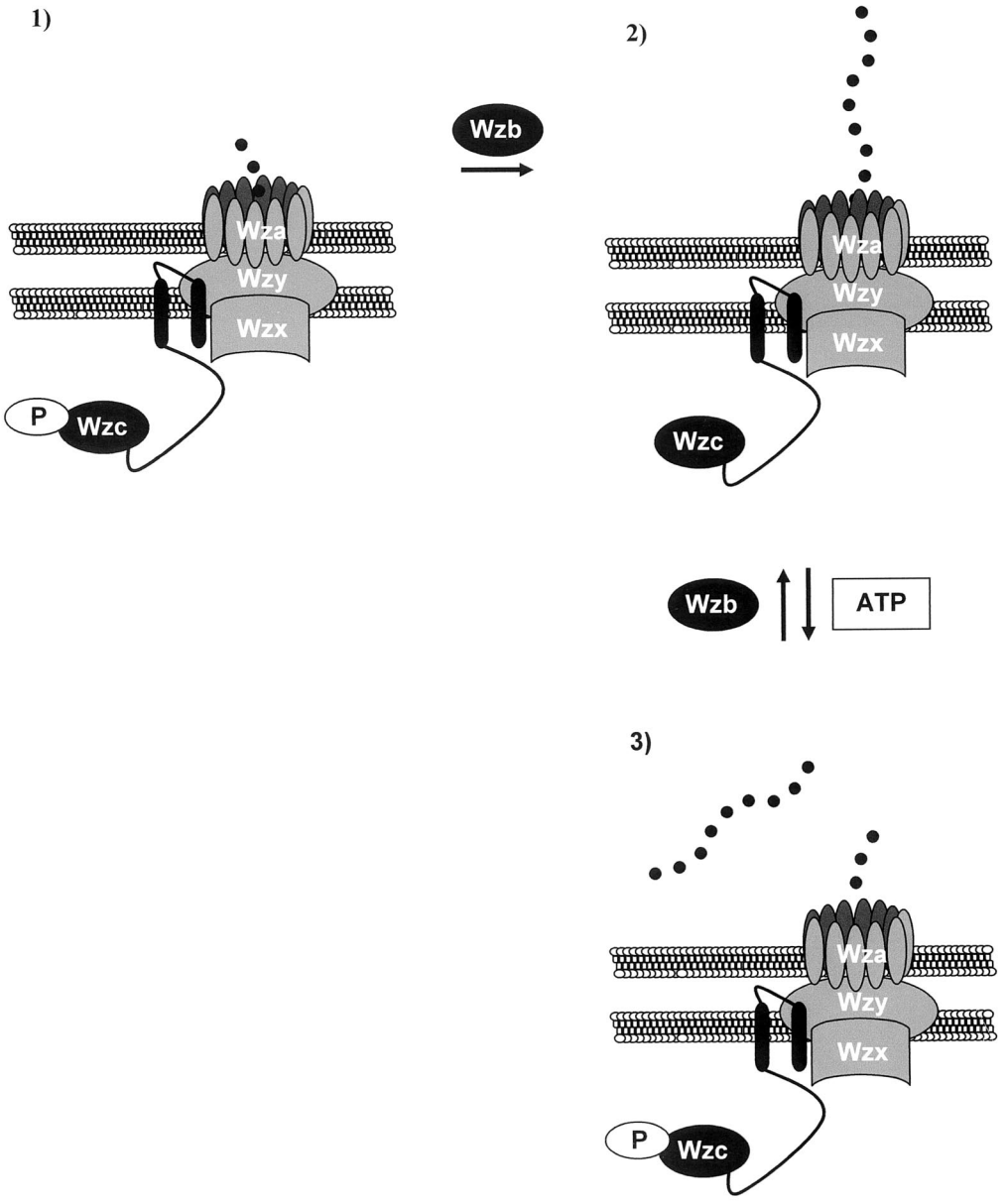

FIG. 8. Cartoon suggesting the role of Wzc phosphorylation in emulsan biosynthesis. (1) Phosphorylation of Wzc prevents polymerization and translocation of emulsan. (2) Wzb dephosphorylates Wzc. Dephosphorylated Wzc allows polymerization and translocation of emulsan. (3) Wzc autophosphorylates. Release of emulsan and the beginning of another round of emulsan polymerization and translocation. This determines the size of the polysaccharide. On the basis of sequence homology, Wzx is considered to catalyze the translocation of the membrane-bound repeat unit through the inner membrane, Wzy has been implicated in polymerization of the repeat unit on the periplasmic side of the cytoplasmic membrane, and Wza has been proposed to form an outer membrane pore through which the capsular antigen is translocated (25). ●, monomer of emulsan.

either of the two genes gave rise to an emulsan-defective phenotype. Moreover, Wzc from RAG-1 was found to be an ATP-dependent autophosphorylating enzyme which is dephosphorylated by Wzb. These properties are consistent with those of other Wzc homologues (14, 20, 38). It should be noted, however, that the autophosphorylation activity of purified Wzc was considerably lower than that reported for Etk, a Wzc homologue of *E. coli* (20). In that case the function of the homologue is unknown (20, 41). As discussed below we predict that Wzc is a member of a complex. It is possible, therefore, that the enzyme is stabilized by other components in that complex and loses activity in their absence. Wzb also showed a lower rate of dephosphorylation. The slow dephosphorylation of Wzc by Wzb is in line with the observations previously reported by others (38, 39).

A tyrosine cluster at the C terminus of MPA1 family proteins has been suggested to be the site of phosphorylation (23, 41). Our results are consistent with this suggestion, since mutants lacking the five tyrosine residues were no longer phosphorylated. Recently, it was proposed that a tyrosine residue

upstream of the C-terminal tyrosine cluster is involved in a cooperative two-step mechanism in which one molecule of Wzc, phosphorylated at the upstream tyrosine, is transferred to the tyrosine cluster at the C terminus of a second Wzc molecule (13). This suggestion was based on the finding that mutants lacking the tyrosine cluster still accumulated small amounts of phosphorylated Wzc. If this upstream tyrosine residue conserved in Wzc of RAG-1 (Y_{575}) was phosphorylated, it should have been detected in the Wzc mutants lacking the C-terminal tyrosine cluster. However, this mutant showed no cross-reaction with antiphosphotyrosine antibodies. It is, however, possible that a more sensitive mode of detection such as labeling with [γ-^{32}P]ATP would have detected this phosphotyrosine. Experiments to reveal the function of the C-terminal tyrosine cluster and its relation to the upstream tyrosine residue are currently in progress.

Mutants LN503 and LN504 showed only low levels of cell-bound galactosamine yet produced abnormally high levels of a high-molecular-mass galactosamine-containing extracellular polysaccharide. It is likely that this polymer accounts for the highly viscous morphology in colonies of these mutants. Mutants of *E. coli* defective in phosphorylation of Wzc also showed low levels of cell bound K30 antigen (41). Mucoid colonial morphology was reported in a mutant of S*treptococcus pneumoniae* lacking a phosphorylation site in CpsD required for biosynthesis of the capsular polysaccharide in this strain (23). This mutant still produced parental levels of capsule. No data were presented in either of the two cases regarding extracellular polysaccharides in the cell-free broth. In contrast to findings reported here, production of high-molecular-mass exopolysaccharide succinoglycan was severely reduced in mutants of *Sinorhizobium meliloti* defective in phosphorylation of ExoP (26), perhaps implying a different mode of polymer synthesis, assembly, and export than in the gram-negative RAG-1.

Hypothetical model for involvement of phosphorylation in emulsan biosynthesis. Fig. 8 is a cartoon describing our prediction of how Wzb and Wzc might regulate emulsan biosynthesis. The proteins appearing in this cartoon are suggestions only. There is nothing to preclude the possibility of other proteins such as transglycosylases also being part of the complex.

Wzc has a periplasmic domain that is predicted to form coiled-coils, a feature important for protein-protein interactions (24). Accordingly, we assume that Wzc may be part of a larger complex involved in emulsan translocation as previously suggested for other Wzc homologues (24, 40). However, in addition to showing a role in translocation, our model also predicts a role in polymerization and release of the emulsan polymer (Fig. 8). Implicit in this model is a structural-mechanical role for both the phosphorylated and dephosphorylated forms of Wzc. The model is based on two observations. (i) Mutants defective in Wzb are also defective in production of both cell-bound as well as cell-free emulsan. This is a condition in which Wzc would be expected to accumulate as a completely phosphorylated protein. (ii) Mutants in Wzc lacking the phosphorylation sites produce no cell-associated emulsan but do produce a very high molecular mass galactosamine-containing polysaccharide in the supernatant.

In this model the nonphosphorylated state of Wzc permits the complex to initiate polymerization and transport of the biopolymer to the outside of the cell (Fig. 8). Previous studies have demonstrated that polymer synthesis accompanies polymer release (32). The subsequent phosphorylation of Wzc is thought to bring about the cessation of polymerization and transport, thereby establishing the size of extracellular emulsan (Fig. 8). The putative involvement of Wzc in regulating emulsan polymerization explains the resemblance of Wzc in *E. coli* with Wzz, which is the chain length regulator of Wzy dependent O-antigens (7).

The model presented here predicts a role for phosphorylation and dephosphorylation of Wzc in regulating polymerization, translocation, and release of the emulsan polymer. Two factors—the activity of Wzb as well as the availability of ATP, the substrate for autophosphorylation of Wzc—might be expected to regulate the system in a transient fashion. In this regard, only very small quantities of emulsan are produced when the cells are grown in rich medium (11). The production of many other exopolysaccharides, such as colanic acid (12), has also been found to be reduced in rich medium. Currently little is known about other factors, either environmental or intracellular, which could affect levels of Wzc phosphorylation. Finally, the ability to manipulate both the size and therefore the viscosity of biopolymers by altering the phosphorylation-dephosphorylation system logically may offer a unique approach to the production of highly viscous useful biopolymers in the future.

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