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Overproduction of Exopolysaccharides by an *Escherichia coli* K-12 *rpoS* Mutant in Response to Osmotic Stress▽†

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The *yjbEFGH* operon is implicated in the production of an exopolysaccharide of an unknown function and is induced by osmotic stress and negatively regulated by the general stress response sigma factor RpoS. Despite the obvious importance of RpoS, negative selection for *rpoS* has been reported to take place in starved cultures, suggesting an adaptive occurrence allowing the overexpression of RpoD-dependent uptake and nutrient-scavenging systems. The trade-off of the RpoS-dependent functions for improved nutrient utilization abilities makes the bacterium more sensitive to environmental stressors, e.g., osmotic stress. In this work, we addressed the hypothesis that overinduction of genes in *rpoS*-deficient strains indicates their essentiality. Using DNA microarrays, real-time PCR, and transcriptional fusions, we show that genes of the *wca* operon, implicated in the production of the colanic acid exopolysaccharide, previously shown to be induced by osmotic stress, are also negatively controlled by RpoS. Both exopolysaccharides in the synthesis of which *yjb* and *wca* are involved are overproduced in an *rpoS* mutant during osmotic stress. We also show that both operons are essential in an *rpoS*-deficient strain but not in the wild type; promoters of both operons are constitutively active in *yjb rpoS* mutants; this strain produces extremely mucoid colonies, forms long filaments, and exhibits a reduced growth capability. In addition, the *wca rpoS* mutant's growth is inhibited by osmotic stress. These results indicate that although induced in the wild type, both operons are much more valuable for an *rpoS*-deficient strain, suggesting that the overproduction of both exopolysaccharides is an adaptive action.

The role of the general stress response sigma factor RpoS (σ^{38}) in *Escherichia coli*'s physiology has been extensively studied. While most studies have focused on the characterization of genes positively controlled by RpoS and on the physiological implications of the underexpression of these genes in *rpoS*-deficient strains (22, 61), only a few refer to genes negatively regulated by RpoS and to the consequences of their overexpression.

Inactivation of *rpoS* is common; mutations in the *rpoS* gene have been detected among laboratory bacterial stocks (25, 52), as well as in environmental and clinical isolates of pathogenic and commensal enteric bacteria (1, 43), suggesting that under certain conditions, the loss or attenuation of RpoS activity may be of adaptive value. Other reports indicated that the *rpoS* gene tends to undergo frequent mutations that lead to loss of activity and that the mutated forms appear to spread and become dominant in glucose-limited chemostat cultures (39) or during incubation in stationary phase (5, 66). It was also shown that the rate of *rpoS* mutations spreading throughout the population decreased when osmotic stress was applied to the system (14, 28). The selection pressure on the *rpoS* gene is thought to be largely due to a competition between the sigma factors RpoS and RpoD for the limited number of RNA polymerase core subunits (13, 32). This suggests that *rpoS* inactivation may be beneficial to a starved cell due to the overexpression of RpoD-dependent nutrient-sensing and uptake systems.

It was previously shown (24, 44) that among *E. coli* genes shown to be significantly induced by osmotic stress, one gene, *yjbF*, stood out in that the promoter that drives its induction appeared to be negatively regulated by RpoS. This gene, a member of a four-gene operon (*yjbEFGH*), was listed (15) among genes that are positively dependent upon RcsC, the inner membrane sensor kinase of the Rcs phosphorelay system. The Rcs system controls a variety of physiological functions in prokaryotes, such as extracellular polysaccharide (EPS) synthesis (27, 42, 51, 58), biofilm formation (10, 15, 41), cell division (3), and motility (17, 53). It has been shown (16) that the four genes of the *yjbEFGH* operon are transcribed together, that this operon is induced during growth on solid surfaces, and that it is involved in the production of an unknown EPS.

E. coli K-12 possesses five known sets of genes promoting EPS production: (i) genes involved in O-antigen synthesis (46); (ii) the *wca* operon, responsible for colanic acid (CA)-EPS synthesis (19); (iii) the *pgaABCD* operon which encodes genes involved in the production of poly-β-1,6-*N*-acetyl-D-glucosamine (PGA), a polysaccharide shown to be crucial for biofilm formation (59); (iv) the *dfc* pseudo-operon (comprised of *gfcABCDE*, *etp*, and *etk*), responsible for the production of type IV capsule in *E. coli* O127:H6 (this operon is nonfunctional in *E. coli* K-12 due to the presence of an IS*1* element in its promoter region [40]); and (v) the *yjbEFGH* operon, a paralogue of *dfcABCD* (15).

We propose that overinduction of the *yjb* operon and, possibly, that of other genes involved in EPS production may be an adaptive response to osmotic stress in an *rpoS*-deficient background and that these overinduced genes are of special value for an

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference(s)
Strains		
MG1655	F^- λ^-	Laboratory stock
QC2410	MG1655 *rpoS*::Tn*10*	31
MK1399	MG1655 Δ*yjbEFGH*(*kanR*)	This work
MK1310	MG1655 Δ*yjbE*(*kanR*)	This work
MK1351	MG1655 ΔP_{wza}(*kanR*) (allele introduced from MS1651 [40])	This work
MK2499	QC2410 Δ*yjbEFGH*(*kanR*)	This work
MK2431	QC2410 Δ*yjbE*(*yjbE*::*kanR*)	This study
MK2411	QC2410 ΔP_{wza}(*kanR*)	This work
CF6343	MG1655 Δ*lacIZ*(MluI)	8
SK1150	CF6343 *rcsA*::*kanR*	17
SK1158	CF6343 *rcsB*::Tn*10*	8
SK1154	CF6343 *rcsC*::Tn*10*	8
MS1651	EPEC O126:H6 ΔP_{wza}(*kanR*)	40
DY378	W3110 λ*cI857*Δ(*cro-bioA*) (permissive strain for gene inactivation)	64
Plasmids		
pDEW201	Promoterless; *luxCDABE ori* pMB1(pBR322) *rop ampR*	56, 57
pDEW/*yjbF* (pDEW609)	pDEW201; *yjbF′*::*luxCDABE* (*pgi-yjbF*)	56, 57
pDEW/*wza*	pDEW201; *wza′*::*luxCDABE*	This study
pDEW/*osmY*	pDEW201; *osmY′*::*luxCDABE*	56, 57
pDEW/*lon*	pDEW201; *lon′*::*luxCDABE*	56, 57
pKD13	Template plasmid for *kanR* amplification	11
pES2	*recA′*::*gfpUV ori* pUC *ampR*	45
pATC400	pBR322 $rcsA^+$ *ampR*	54

rpoS-deficient strain. Our results indicate that alternative stress response strategies may come into play in the absence of RpoS or when its activity is diminished, allowing the cells to survive and proliferate even without its general stress protection.

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study are listed in Table 1. All strains were grown in Luria-Bertani medium (LB broth; 5 g $liter^{-1}$ yeast extract, 10 g $liter^{-1}$ Bacto-tryptone, and 5 g $liter^{-1}$ NaCl) supplemented with the appropriate antibiotics (100 μg ml^{-1} ampicillin, 10 μg ml^{-1} tetracycline, 50 μg ml^{-1} kanamycin) to an optical density at 600 nm (OD_{600}) of 1 to 2 (Spekol 1200; Analytikjene, Germany), mixed with 50% glycerol, and kept at −80°C. Cells were recovered from freezing by overnight growth on LB agar plates. Plates were kept for up to 1 month at 4°C.

Detection of *rpoS* and *wca* mutants. RpoS deficiency was routinely checked qualitatively by a catalase activity assay. A drop of 5 μl of 32% H_2O_2 was placed on a colony; immediate vigorous bubbling indicated wild-type RpoS activity (39), and lack of bubbling was interpreted as RpoS deficiency. Deficiency of *wca* (ΔP_{wza}) was tested by transforming the strain with plasmid pATC400 ($rcsA^+$) (54). Wild-type strains became extremely mucoid (CA-EPS overproduction), and ΔP_{wza} strains did not.

Construction of new inactive alleles. Nonpolar gene deletions were carried out as described previously (11, 64), using the primers listed in Table 2. Briefly, pKD13 (11) was used as a template DNA for PCR with primer pairs 60 bases long designed to amplify the kanamycin resistance gene from the plasmid (20 bases at the 3′ end of each primer) and to undergo a recombination process with the edges of the chromosomal target site (40 bases at the 5′ side of each primer identical to the recombination sites in the chromosome). The ΔP_{wza} allele was directly amplified from the MS1651 genome (40), which was extracted with a DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. PCR (TGradient; Biometra, United States) was carried out with proofreading Bio-X-Act DNA polymerase (Bioline) in the presence of 200 nM template and primers using the manufacturer's reagents and instructions. The ~1,500-bp product was gel purified using a QIAquick gel extraction kit (Qiagen), digested with DpnI to eliminate template plasmid, and then desalted using the same kit. The PCR product was then transformed into DY378 (64), a recombination-permissive strain harboring a lysogenically defective lambda phage. An overnight culture grown at 30°C was regrown to an OD_{600} of 0.6, heat shocked for 15 min to induce the lambda P_L promoter (controlling lambda recombination promoting factors), cooled on ice, washed four times in cold double-distilled water (DDW), and finally resuspended in 1 ml DDW. Aliquots (100 μl) were mixed on ice with 50 μl of the purified PCR product. Electrical DNA transformation was carried out at 2.5 mV (Electro Cell manipulator ECM 935; BTX, United States), and the cells were then grown for 1 h in 37°C prewarmed LB broth. Positive recombinants were selected on LB agar plates supplemented with kanamycin and verified by PCR and sequencing.

Allele transduction by P1 *kc* coliphage. The new inactive alleles were transduced into MG1655 by P1 *kc* transduction as described previously (48). The *rpoS*::Tn*10* allele from QC2410 was also transduced into MK1399, MK1310, and MK1351 to produce the double mutants MK2499, MK2431, and MK2411. Briefly, an overnight donor bacterial strain culture (carrying a defective allele) was regrown in 10 ml LB supplemented with 5 mM $CaCl_2$ for 40 min before the addition of 100 μl P1 *kc* coliphage (produced on MG1655) and then incubated at 37°C with shaking (200 rpm) for 3 h until the culture was completely lysed by the phage. Cells that survived the lysis were killed by chloroform, and cell debris was removed by centrifugation. The supernatant containing the new phages was collected, and various volumes were incubated at 37°C for 40 min with an overnight MG1655 culture suspended in 10 mM $MgSO_4$, 5 mM $CaCl_2$. P1 infection was stopped by adding 100 μl 0.1 M Na_3 citrate to each sample, and positive transductants were selected on LB agar plates supplemented with the appropriate antibiotics.

Cloning of the *wza* promoter. MG1655 genomic DNA (extracted with a DNeasy plant mini kit [Qiagen]) was used as a template for amplifying the *wza* promoter with primers designed to have a calculated annealing temperature of 72°C to its target site and a tail containing the restriction enzyme digestion site followed by 8 mismatched nucleotides at its far 5′ end. pDEW201 (56, 57) was used as an acceptor for the new DNA fragment. DNAs (plasmid and PCR product) were digested with EcoRI and KpnI and desalted by using a QIAquick gel extraction kit (Qiagen). Ligation was carried out with T4 ligase (Roche) according to the manufacturer's instructions after treating the linear vector with shrimp alkaline phosphatase (Fermentas). Chemical transformation into AG1688 competent cells was carried out. Positive plasmids were selected by colony PCR and verified by sequencing.

DNA microarray and rRT-PCR analysis. Single colonies of strains MG1655 and QC2410 (*rpoS*::Tn*10*) grown (37°C) overnight on LB broth were regrown in fresh LB broth containing 0.09 or 0.7 M NaCl for 90 and 180 min, respectively, until the wild-type strain reached an OD_{600} of 0.3. The cultures were then diluted to the density obtained by the *rpoS* mutant (OD_{600} = 0.15), and 50 ml of each strain was subjected to total RNA extraction using an RNeasy midi kit (Qiagen) and DNase I (Qiagen) treatment. The RNA concentration was determined by using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Twenty micrograms of total RNA was reverse transcribed with a poly(T_{18}) primer using a RevertAid first-strand cDNA synthesis kit (Fermentas), and mRNA expression was assayed by using an Affymetrix GeneChip *E. coli* antisense genome array (Weizmann Institute of Science [Rehovot, Israel] MicroArray Unit) according to the manufacturer's instructions. The DNA microarray results were confirmed by relative real-time PCR (rRT-PCR) analysis (30). Twenty-five nanograms of cDNA was mixed with Sybr green reaction mixture (ABI) and assayed with an ABI Prism 7000 sequence detection system (Applied Biosystems) for the relative quantification of the abundance of each of the following genes: *yjbE*, *yjbF*, *yjbG*, *yjbH*, *wzc*, *gmd*, *manC*, *wcaH*, *wcaD*, and *rcsA*. Two additional genes that were similarly amplified were the RpoS-dependent *osmY* that served as a negative control and the RpoS-independent housekeeping gene *rpmA* that served as a reference. The latter gene yielded average threshold cycle (C_T) values (n = 3) of 17.8 ± 0.7 (mean ± standard deviation) and 17.5 ± 0.6 for MG1655 and QC2410, respectively. The reaction conditions were as follows: 50°C for 2 min, hot start at 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. A dissociation test (12) was performed at the end of each run to ensure a single PCR product in each reaction mixture. Results were obtained with the SDS analysis program (ABI) using a relative quantification algorithm with automatic C_T calculation. A ΔC_T was calculated for each gene as $\Delta C_T = C_T$(studied gene) − C_T(*rpmA*). $\Delta\Delta C_T$ was calculated in two different ways. (i) RpoS dependency was calculated with the equation $\Delta\Delta C_T = \Delta C_T$(*rpoS*::Tn*10*) − ΔC_T(MG1655). (ii) The relative expression of *wca* genes

TABLE 2. Primers used in this study

Primer name, purpose	Sequence (5′ → 3′)[a]	Use
Gene inactivation		
Pwza-F	CGTTGGTGATCGCCTGTTGACCGG	Transfer of P_{wza} allele from MS1651 to MG1655
Pwza-R	GCCGTTCGTTCAGCTCCATCGTGG	Transfer of P_{wza} allele from MS1651 to MG1655
yjbE-F	ATTTTTGCCATATCTGCGCTTGCGGCGACTTCTGCGT GGGCGTGTAGGCTGGAGCTGCTTC	Inactivation of *yjbEFGH* and *yjbE*
yjbE-R	GTCGTGGTTGTGGTGGTCCCGGTATTAGAACCA TCACCGCATTCCGGGGATCCGTCGACC	Inactivation of *yjbE*
yjbH-R	CGCACGGCTGCGTGTCGGGCCAGACGAGAAGAGAT CCAACGGTACATTCCGGGGATCCGTCGACC	Inactivation of *yjbEFGH*
Cloning (pDEW201 based)		
wza-F	TATACATGGAATTCCGATTAACCCGGCCCAGATAGACG	EcoRI-mediated ligation
wza-R	ATCAGTGGGGTACCCCCCCGCCCTCGCCTTTCAGCG	KpnI-mediated ligation
rRT-PCR analysis		
yjbE-F	TTTGCCATATCTGCGCTTG	
yjbE-R	TGGTGGTCCCGGTATTAGAA	
yjbF-F	AAGCGACCTGCACTCATTCT	
yjbF-R	AGGCCAGCACCACAAATAAC	
yjbG-F	TATTGTCGCGTTGCTTTTGA	
yjbG-R	CCAGCTCTTCGCTAATCACC	
yjbH-F	GCTTGCTACGGCGAAACATA	
yjbH-R	GGGAAGAGTTGCACTGAAGC	
wzc-F	CCTCGATATTGCAGTGAGCA	
wzc-R	CGCTGCTCAGGGTGTAGTTT	
wcaD-F	CCCATCACCATCGTCACTTT	
wcaD-R	CCACACCATGCCAATAATGA	
gmd-F	ATGGCGACCTGAGTGATACC	
gmd-R	TTTCTTTTTCCAGACCGAGGA	
wcaH-F	AGGAAGACTTTGCCACGGTA	
wcaH-R	CTTCCAGCGTTTCGTCTTTC	
manC-F	ATGAGCAGCACCGCTTTATT	
manC-R	CCGCCAATACCAGCATTAAC	
rcsA-F	CGACATTGAAACCGTTGATG	
rcsA-R	GCAATTGCCATAAAAACGAT	
osmY-F	TGCTGGCTGTAATGTTGACC	
osmY-R	TCGGTGCTCTTGATGTTGTC	
rpmA-F	TAACGGTCGCGATTCAGAAG	
rpmA-R	GCAAACAGAGTGTGGTCACG	

[a] Underlined letters indicate restriction enzyme digestion sites.

compared to *yjb* genes was calculated with the equation $\Delta\Delta C_T$ = [averaged ΔC_T(*wzc*, *gmd*, *manC*, *wcaH*, *wcaD*)] − [averaged ΔC_T(*yjbE*, *yjbF*, *yjbG*, *yjbH*)]. RQ (relative RNA quantification) was calculated as RQ = $2^{-\Delta\Delta C_T}$ for the results of each experiment. Results are presented as averaged RQ values ($n = 3$).

Bioluminescence monitoring of promoter induction. Induction of promoters was routinely monitored by following the luminescence of *E. coli* strains bearing pDEW201-derived plasmids (56, 57) (Table 1) harboring promoter::*luxCDABE* (*Photorhabdus luminescens luxCDABE* bioluminescence genes) transcriptional fusions in 96-well microtiter plates. All experiments were carried out in LB broth supplemented with 0.09, 0.3, 0.5, or 0.7 M NaCl. To ensure similar plasmid copy numbers (24), the lag phase was eliminated by initiating the experiments with a growing culture. A single colony was grown to an OD_{600} of 0.5 on LB broth supplemented with the appropriate antibiotic at 37°C, diluted 1:10 in fresh medium, and then regrown to an OD_{600} of 0.2. Fifty microliters of bacterial culture was added to the wells of an opaque, white, clear-bottom 96-well microtiter plate already containing 50 μl fresh LB broth supplemented with either twice the required NaCl concentration or 0.09 M NaCl (basal growth medium concentration). The plate was then sealed with a transparent cover and incubated at 37°C for 300 min in a microtiter plate reader (Victor^2; Wallac, Finland). Bioluminescence (reported in the instrument's arbitrary relative light units) and OD_{600} were measured at intervals of 15 min, each following a 5-s shaking. For several hours following exposure to NaCl in the microtiter plate, the OD_{600} of the NaCl-exposed cells did not change. Bioluminescence data were normalized to a uniform cell density by dividing the measured light intensity (relative light units) by the OD_{600} value measured in the same well at the same time point. Maximum bioluminescence refers to the peak of activity obtained in the course of 300 min of measurements.

EPS-related phenotype characterization. Single colonies were spread on LB agar plates supplemented with either 0.09 or 0.7 M NaCl with or without 150 μg ml^{-1} Congo red (16) and allowed to grow for 24 h at 37°C. Plates were photographed over a black background (without Congo red) or a light screen (with Congo red) with an Olympus Stylus-770 SW digital camera. A mucoid appearance indicated overproduction of CA-EPS by the *wca* operon; red-stained cells indicated the presence of the EPS driven by the *yjb* operon.

Light/fluorescence microscopy. Five microliters of culture was mounted on a microscopic slide or in a Neubauer counting chamber (400-μm^2 by 10-μm-deep Clay Adams; Becton Dickinson, NJ), covered with a coverslip, and analyzed with an epifluorescence microscope (Axivert 135TV; Zeiss, Germany) or a light microscope (Eclipse N100; Nikon, Japan). Photographs were acquired with a mounted Canon PowerShot A95 digital camera.

Total carbohydrate determination. Overnight colonies grown on LB agar plates supplemented with 0.7 or 0.09 M NaCl were collected and suspended in 2% Na_2SO_4, vigorously mixed for 10 min, and calibrated to a uniform cell concentration of 10^{10} ml^{-1}. The following analytical protocol was used (65): 500

μl of cells was extracted by the addition of 800 μl chloroform, vigorous mixing, and phase separation by centrifugation (14,000 rpm for 10 min). A 300-μl amount of the upper (aqueous) phase was mixed with 200 μl DDW and with 1 ml anthrone reagent (Sigma) stock solution (1 mg ml^{-1} in concentrated H_2SO_4 [95 to 98%, wt/vol]). The mixture was incubated at 90°C for 10 min, and the OD_{630} of the sample was determined. Total carbohydrate content was calculated from a trehalose (Sigma) calibration curve.

Characterization of growth capability. Single colonies were grown on LB broth supplemented with the appropriate antibiotics to an OD_{600} of 0.5, diluted 1:10 into fresh LB broth supplemented with a final concentration of either 0.7 or 0.09 M NaCl, and incubated at 37°C with shaking (200 rpm) for 6 h. The OD_{600} was monitored at 2-h intervals. To verify the correlation of OD measurements to the real cell concentration, a determination of growth was also performed at some time points by direct microscopic counts (Eclipse N100; Nikon, Japan).

RESULTS

Genes of the *wca* operon are induced by osmotic stress and are repressed by RpoS. As previously reported (24, 44), the promoter regulating the induction of the *yjbEFGH* operon is activated by osmotic stress and is repressed by RpoS. In an attempt to identify additional EPS synthesis-related genes that may be coregulated with *yjbEFGH*, we have screened for genes that share these two regulatory features: induction by NaCl and overinduction in an *rpoS*::Tn*10* mutant.

For this purpose, the MG1655 wild-type strain and its *rpoS*::Tn*10* derivative were both grown in LB supplemented with either 0.09 or 0.7 M NaCl until the wild-type cultures reached an OD_{600} of 0.3. All samples were then diluted to the same OD and subjected to total RNA extraction, followed by whole-cell DNA microarray analysis. Sampling times were chosen based on the activity of the *yjbF′*::*luxCDABE* transcriptional fusion in each strain in each NaCl dose; after 180 min, it was an order of magnitude higher in the *rpoS* mutant than in the wild type with both grown in LB broth supplemented with 0.7 M NaCl.

Three members of the *yjb* operon, *yjbE*, *yjbF*, and *yjbH*, displayed osmotic-stress response ratios of 33 (result of a very low background), 3.9, and 2.2 (Table 3). In the few previous reports of DNA microarray studies that have investigated the response of *E. coli* to osmotic shock (e.g., reference 60), members of the *yjb* operon have not been listed among the osmotically regulated genes. This may be attributed to the lower NaCl doses (<0.5 M) applied in these studies, as well as to the short duration of exposure (<30 min). In the present study, based on the observed induction characteristics of the *yjbF′*::*luxCDABE* transcriptional fusion, the experimental conditions were harsher. In our DNA microarray, *yjbE*, *yjbF*, and *yjbH* displayed RpoS dependency ratios of 1.7, 1.6, and 1.7, respectively; the RpoS dependency threshold ratio was therefore set at 1.5.

Among 774 genes that demonstrated ≥2-fold overexpression both in the wild type and in the *rpoS* mutant in the presence of 0.7 M NaCl, only 187 also displayed a ≥1.5-fold-increased level of expression in the *rpoS* mutant in comparison to their level of expression in the wild type (see Table S1 in the supplemental material). Also selected by this double criterion were 14 out of the 21 known genes of the *wca* operon, displaying osmotic-shock response ratios of 3.2 to 27 and RpoS dependency ratios of 1.5 to 2.8 (Table 3). The *wca* operon was previously reported (49) to be induced by osmotic stress, but its negative dependence on *rpoS* was not demonstrated. A few additional RcsC-dependent genes, as defined previously (15), behaved similarly, including *osmB*, *rcsA*, *ugd*, *ykfE*, *ygaC*, *ymgD*, *yhaL*, and *yhaK* (see Table S1 in the supplemental material). As previously reported (18), another functional EPS production operon, *pgaABCD*, is also induced by osmotic stress. In our experiment, its members displayed osmotic-stress response ratios of 1.6 to 6.1 but exhibited neither positive nor negative dependency upon RpoS.

TABLE 3. Expression ratios obtained by DNA microarray analysis and rRT-PCR of gene members of various EPS production systems[a]

Gene	Microarray analysis ratio		rRT-PCR RpoS dependency ratio[c]	Comment
	Osmotic-stress response[b]	RpoS dependency[c]		
yjbE	33.2	1.7	7.1 ± 0.4	*yjb* operon
yjbF	3.9	1.6	5.3 ± 0.4	
yjbG	1.9	1.8	4.1 ± 0.4	
yjbH	2.2	1.7	4.1 ± 0.3	
wza	5	2.7	ND	*wca* operon
wzb	3.8	1.7	ND	
wzc	4.4	2	5 ± 0.3	
wcaA	3.1	2.1	ND	
wcaB	9	2.7	ND	
wcaC	8.8	1.9	ND	
wcaD	27	2.6	4.4 ± 0.7	
wcaG	4	1.8	ND	
gmd	5.1	1.6	7.9 ± 0.3	
wcaH	5.7	2.2	8.4 ± 0.4	
wcaI	2.6	1.7	ND	
manC	18	1.4	6.1 ± 0.5	
wzxC	4.3	1.8	ND	
wcaK	4	1.5	ND	
wcaM	3.2	1.6	ND	
rcsA	5.9	1.9	4.1 ± 0.7	Transcription activator of *wca*/*yjb* operons
osmY	6.3	0.3	0.3 ± 0.4	RpoS dependent

[a] Expression ratios for the wild type and its *rpoS* mutant were obtained following growth in LB supplemented with either 0.7 or 0.09 M NaCl.
[b] MG1655 (0.7 M NaCl)/MG1655 (0.09 M NaCl).
[c] *rpoS*::Tn*10* (0.7 M NaCl)/MG1655 (0.7 M NaCl). ND, not determined.

The apparently similar induction characteristics (osmotic stress and RpoS dependency) of *yjb* and *wca* were confirmed by relative real-time PCR analysis, applying the same sample preparation procedure employed for the DNA microarray analysis. The RpoS-dependent gene *osmY* served here as a negative control. Indeed, as also shown in Table 3, all genes tested (four members of the *yjb* operon, five members of the *wca* operon, and their common regulator *rcsA*) displayed RpoS dependency ratios ranging from 4 to 8, confirming the general trend obtained by the DNA microarray analysis. As expected, the RpoS-dependent gene *osmY* was induced by NaCl but was repressed in the *rpoS* mutant.

The *yjb* and *wca* promoters display similar induction patterns. To study the induction characteristics of the two EPS biosynthesis operons, *wca* and *yjb*, we have cloned the promoter of *wza*, the first gene of the *wca* operon (50), to produce a *wza′*::*luxCDABE* transcriptional fusion. The activity of this construct was compared to that of the previously described (24) *yjbF′*::*luxCDABE* in the presence of different NaCl concentrations, both in the wild type and in the *rpoS* mutant (Fig. 1). As in the rRT-PCR analysis, the RpoS-dependent promoter of *osmY* (*osmY′*::*luxCDABE*) (56, 57) served as the control. As is clearly evident from the first four panels (A to D)

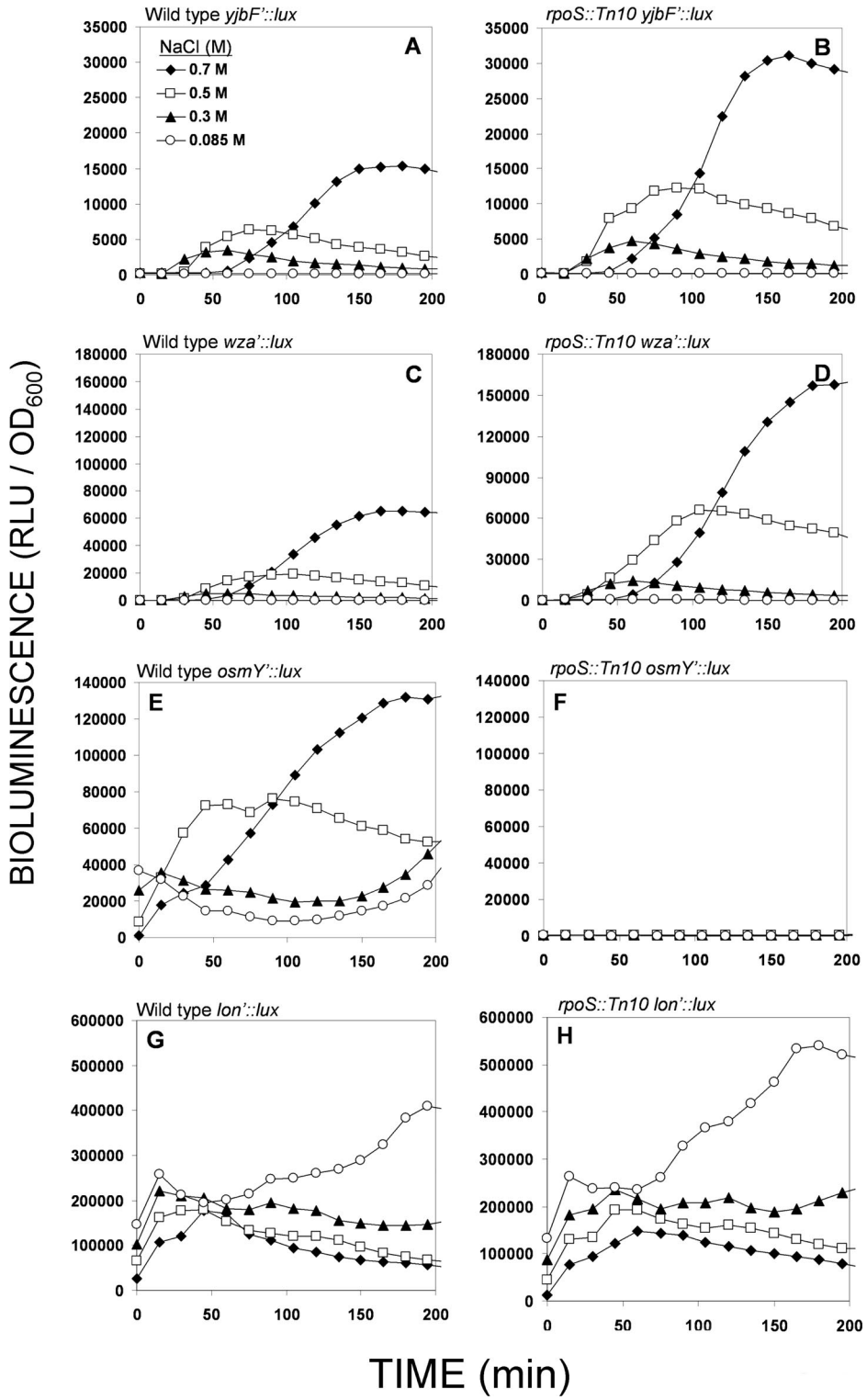

FIG. 1. Activities of *yjbF'*::*luxCDABE* (A, B), *wza'*::*luxCDABE* (C, D), *osmY'*::*luxCDABE* (E, F), and *lon'*::*luxCDABE* (G, H) transcriptional fusions in response to different NaCl concentrations in the wild-type strain (A, C, E, and G) and in the *rpoS*::Tn*10* mutant (B, D, F, and H). RLU, relative light units.

of Fig. 1, the *yjb* and *wca* promoters exhibit similar patterns of induction in response to three different NaCl concentrations, both in the wild type (Fig. 1A and C) and in the *rpoS*::Tn*10* mutant (B and D); furthermore, the activation of both promoters was similarly enhanced by the *rpoS* mutation and peaked at the same NaCl concentration (0.7 M) (Fig. 2A). The only difference observed in the induction of the two promoters was in the intensity; the bioluminescence exhibited by the *wza'*

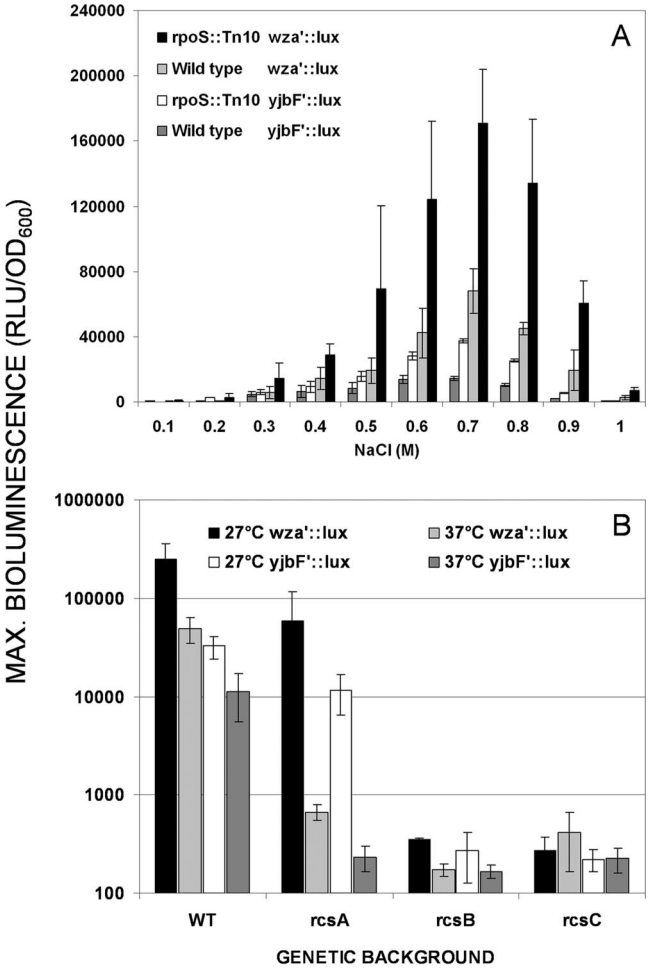

FIG. 2. (A) NaCl dose dependency of *yjbF'*::*luxCDABE* and *wza'*::*luxCDABE* induction in the wild-type strain and the *rpoS*::Tn*10* mutant. (B) Induction of *yjbF'*::*luxCDABE* and *wza'*::*luxCDABE* transcriptional fusions in response to 0.7 M NaCl in *rcsA*, *rcsB*, and *rcsC* mutants and their ancestral wild type at 27°C and 37°C. Error bars show standard deviations. Max, maximum; RLU, relative light units.

fusion was approximately fivefold higher in both the wild type and the *rpoS* mutant. The same was true for the rRT-PCR analysis; the averaged RQ values (see Materials and Methods) for *wca* gene expression compared to the expression of *yjb* genes were 4.7 ± 2.2 and 5.1 ± 1.1 for the wild type and the *rpoS* mutant, respectively. As expected, the *osmY* promoter exhibited a strong positive response to osmotic stress, but its activity was strongly inhibited in the *rpoS*::Tn*10* mutant (Fig. 1E and F). An RpoS-independent gene promoter (*lon*, an RpoH-dependent gene) exhibited similar activity patterns and intensities in both strains (Fig. 1G and H).

The *yjb* operon was listed (14) among genes controlled by the Rcs phosphorelay system in an RcsA-dependent manner (16). The same authors also pointed out that a sequence highly similar to the RcsAB box (62) is located in the *yjbE* promoter region. We confirmed these findings by assessing the osmotic (0.7 M NaCl) induction and activity of the *yjbF'*::*luxCDABE* and *wza'*::*luxCDABE* transcriptional fusions in *rcsA*-, *rcsB*-, and *rcsC*-inactive strains (Fig. 2B). The experiments were conducted at two temperatures, 27°C and 37°C, as it was previously shown (49) that the *wca* operon is more active at the lower temperature. As before, the induction of both promoters exhibited very similar patterns but different intensities. Both were almost completely inhibited in the *rcsC* and *rcsB* mutants, and both displayed a higher activity at the lower temperature in the wild type. In the *rcsA* mutant, the inhibition of activity was temperature dependent: it was significantly inhibited (74-fold for *wca* and 49-fold for *yjb*) at 37°C and only moderately reduced (4.2-fold for *wca* and 2.8-fold for *yjb*) at 27°C.

Overproduction of EPS in *rpoS* mutants. CA-EPS production (mucoid appearance) was unnoticeable in wild-type colonies grown at 37°C on LB agar plates supplemented with either 0.09 or 0.7 M NaCl (Fig. 3A); however, in 0.7 M NaCl, the *rpoS*::Tn*10* mutant produced moderate mucosity. Inactivation of the *wca* promoter on top of *rpoS* mutation (ΔP_{wza} *rpoS*::Tn*10*) appeared to limit growth somewhat but clearly abolished mucosity (Fig. 3A). In a parallel manner, the same was true for the *yjb*-dependent EPS (Fig. 3B) characterized by Congo red staining: it was not observed in the wild type but was apparent in the *rpoS* mutant. Inactivating the *yjb* operon in the *rpoS* mutant (Δ*yjbEFGH* *rpoS*::Tn*10*) suppressed the overproduction of the EPS stained with Congo red and, in addition, gave rise to extreme mucosity. The carbohydrate contents of these cultures (Table 4) support this visual observation. The carbohydrate concentrations of all strains were higher when strains were grown on LB agar plates supplemented with 0.7 M NaCl. The increase in carbohydrate in the wild-type strain may be due to enhanced activity of several EPS production systems known to be induced by osmotic stress: PGA, *yjb*-dependent EPS, and CA-EPS. In the *rpoS* mutant, only the latter two are expected to be overproduced; from the data in Table 4 it may be roughly estimated that the mixture is dominated by the CA-EPS.

Δ*yjb* *rpoS*::Tn*10* double mutants are filamentous. Upon deletion of *yjbEFGH*, the *rpoS*::Tn*10* strain not only lost the red staining described above but also became extremely mucoid (Fig. 3A and B). In addition, the cells elongated into filaments up to 200 μm long (Fig. 4A). These phenotypes were no longer dependent upon osmotic stress and were also observed during growth on regular LB agar plates (0.09 M NaCl). To better visualize this phenomenon, the cells were transformed with a plasmid containing a green fluorescent protein gene (pES2, *recA'*::*gfpUV*) (45). Furthermore, a Δ*yjbE* *rpoS*::Tn*10* double mutant was found to exhibit identical colony (not shown) and cellular (Fig. 4B) appearance. Both phenotypes were negated when this strain was transformed with plasmid pDEW/*yjbF*, which harbors an active *yjbE* gene, confirming the nonpolar deletion of *yjbE*. The cellular aspect of the complemented phenotype is also shown in Fig. 4B.

Activity of both *yjb* and *wca* promoters is enhanced in the Δ*yjbEFGH* *rpoS*::Tn*10* mutant. To explain the NaCl-independent mucoid colony appearance exhibited by the Δ*yjbEFGH* *rpoS*::Tn*10* mutant, the activities of both promoters (*yjb* and *wca*) were assayed in the wild type and its three mutants, Δ*yjbEFGH*, *rpoS*::Tn*10*, and Δ*yjbEFGH* *rpoS*::Tn*10*. The background activity of both promoters in the *rpoS* mutant was five- to sixfold higher than in the wild-type strain, suggesting that the molecular mechanism that promotes these overinductions is osmotic-stress independent. As might be expected, the background activity (in 0.09 M NaCl) of both promoters was much

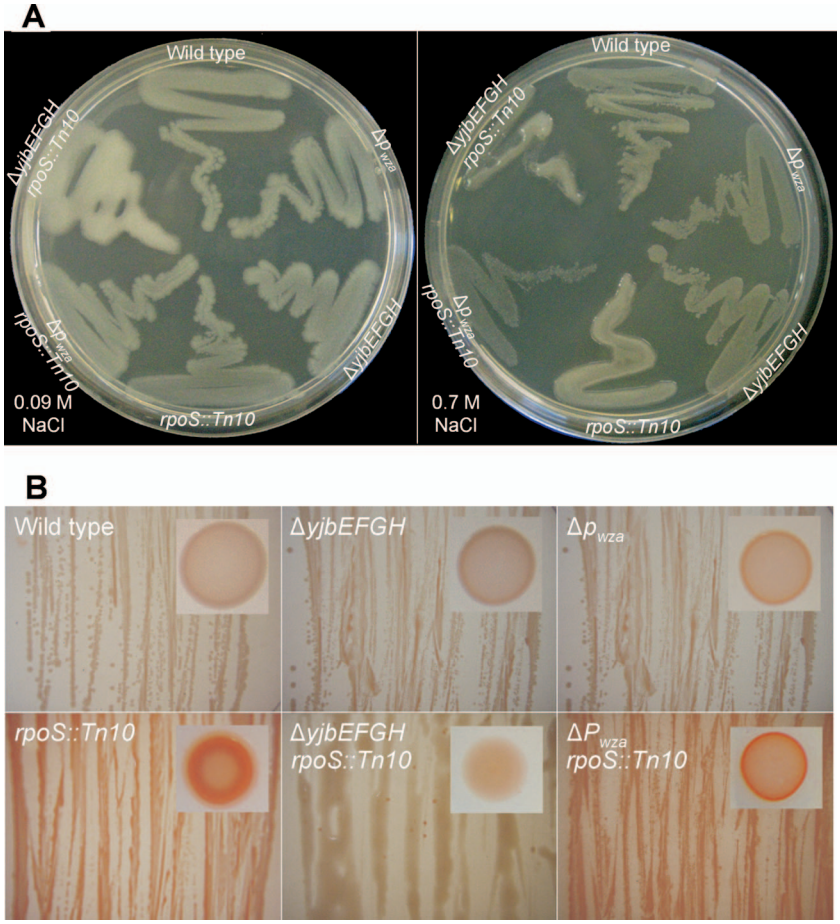

FIG. 3. (A) Appearance of culture after 24 h of growth on LB agar plates supplemented with 0.09 M or 0.7 M NaCl. (B) Congo red staining of cultures grown on LB agar plates supplemented with 0.7 M NaCl. Representative colonies are shown in the insets.

higher in the Δ*yjbEFGH rpoS*::Tn*10* double mutant than in their *rpoS*::Tn*10* ancestor (Fig. 5). When induced by 0.7 M NaCl, the maximal activity was similar to (*yjb*) or twice as high as (*wza*) the background activity. The latter observation appears to be significant in spite of a large standard deviation in the *wza′*::*lux* background measurements. In either case, the response of the double mutant does not fully explain the 10-fold-higher carbohydrate content (CA-EPS) of this strain grown on 0.7 M NaCl (Table 4).

TABLE 4. Carbohydrate content of cells grown on LB agar plates supplemented with different concentrations of NaCl

Genotype	Carbohydrate content ± SD (μg/10^9 cells)	
	0.09 M NaCl	0.7 M NaCl
Wild type	3.4 ± 2.6	11.6 ± 6
ΔP_{wza}	2.7 ± 2.4	9.1 ± 4.5
Δ*yjbEFGH*	2.4 ± 1.4	16.1 ± 9.5
rpoS::Tn*10*	0.9 ± 0.3	393 ± 124[a,b]
ΔP_{wza} *rpoS*::Tn*10*	1.7 ± 1.1	136 ± 16[b]
Δ*yjbEFGH rpoS*::Tn*10*	79 ± 39[a]	806 ± 136[a]

[a] Mucoid.
[b] Congo red positive.

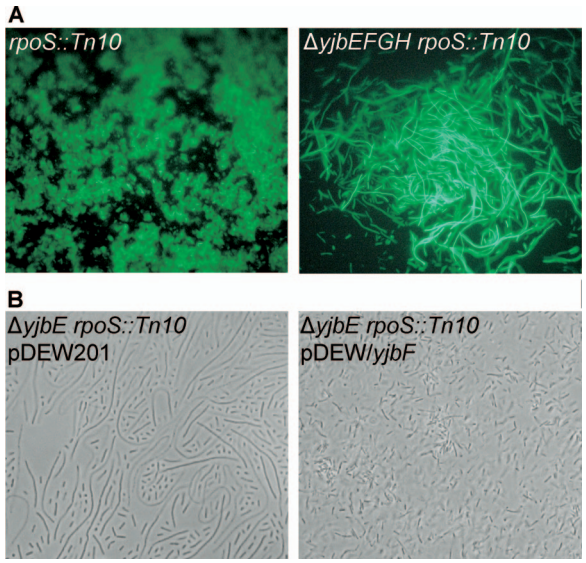

FIG. 4. (A) Fluorescence microscopy of *rpoS*::Tn*10* and Δ*yjbEFGH rpoS*::Tn*10* mutants harboring pES2 (*recA′*::*gfp*). (B) Light microscopy of Δ*yjbE rpoS*::Tn*10* harboring pDEW201 (vector only) or pDEW/*yjbF* (containing *yjbE* upstream of *yjbF′*).

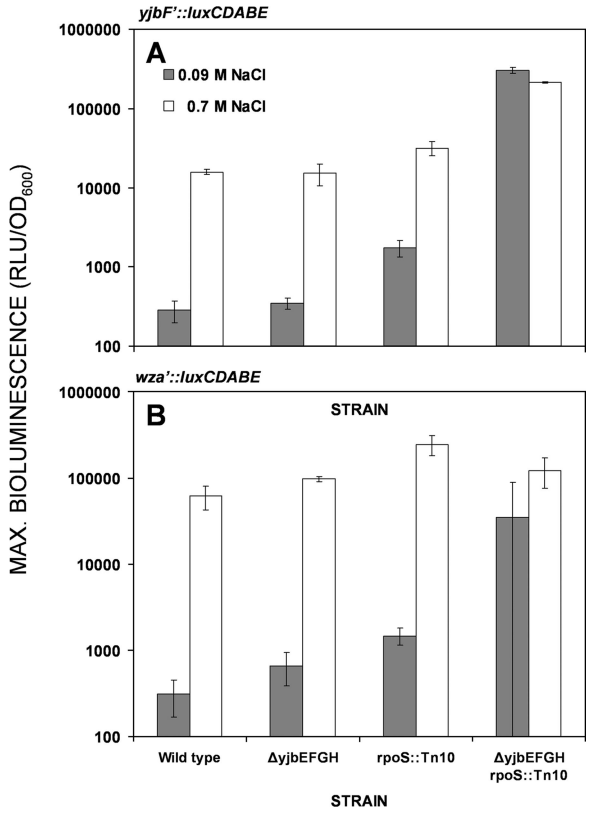

FIG. 5. Maximal activities of the *yjbF'*::*luxCDABE* (A) and *wza'*::*luxCDABE* (B) transcriptional fusions in the wild type and Δ*yjbEFGH*, *rpoS*::Tn*10*, and Δ*yjbEFGH rpoS*::Tn*10* mutants. Error bars show standard deviations. Max, maximum; RLU, relative light units.

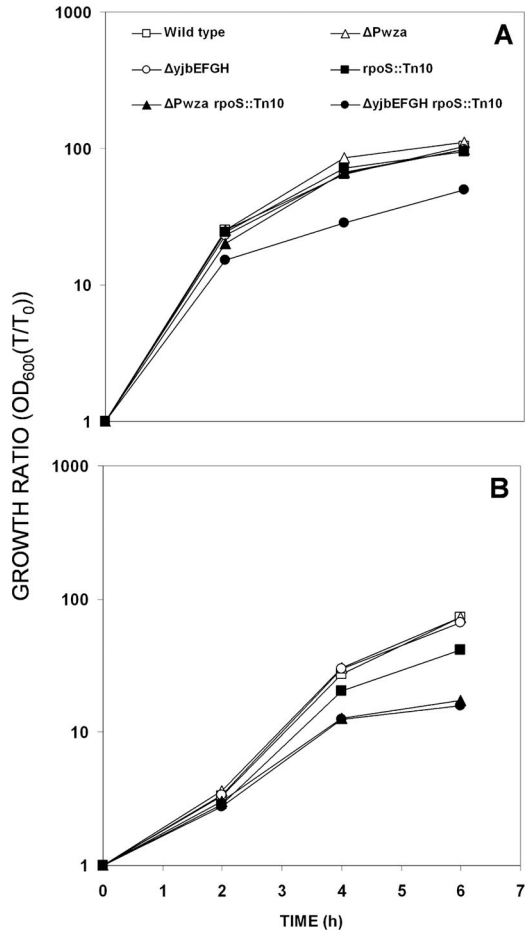

FIG. 6. Growth in LB medium supplemented with 0.09 M (A) or 0.7 M (B) NaCl. The strains are listed in panel A. Results shown are averaged curves of the results of at least three repeat experiments.

Inactivation of either *yjb* or *wca* impaired growth in an *rpoS*-deficient strain. The growth capabilities of all of the strains were tested in LB broth supplemented with 0.09 M (Fig. 6A) or 0.7 M (Fig. 6B) NaCl. While the wild-type strain and the ΔP_{wza} and the Δ*yjbEFGH* mutants exhibited no differences in their growth characteristics, the *rpoS* mutant grew more slowly in the saline medium. Its two derived double mutants, ΔP_{wza} *rpoS*::Tn*10* and Δ*yjbEFGH rpoS*::Tn*10*, exhibited even slower growth: ΔP_{wza} affected growth only in the presence of 0.7 M NaCl, while the effect of Δ*yjbEFGH* was observed also in the low NaCl medium. The effect of osmotic stress on ΔP_{wza} *rpoS*::Tn*10* growth is also shown in Fig. 3B, where this mutant appears to be reduced in comparison to all other strains on the plate.

Direct microscopic counts indicated that the measured OD_{600} values displayed in Fig. 6 are valid for comparisons between the tested strains: an OD_{600} of 1 represents 2.8×10^8 (*rpoS*::Tn*10*), 2.6×10^8 (ΔP_{wza} *rpoS*::Tn*10*), and 2.2×10^8 (Δ*yjbEFGH rpoS*::Tn*10*) cells ml^{-1}. The filamentous phenotype described above (Fig. 4) had started to emerge in the Δ*yjbEFGH rpoS*::Tn*10* double-mutant culture only upon entry to stationary phase and was almost unnoticeable during the course of the experiment.

DISCUSSION

Natural selection for loss of RpoS activity has been shown to occur in the course of nutrient-limited growth and has been also detected among laboratory stocks of enteric bacteria and natural isolates. Since fully or partially inactive *rpoS* alleles are thus common, it may be hypothesized that some aspects of low-RpoS or RpoS-free physiology have evolved to compensate for the loss of the physiological functions that are normally RpoS dependent.

RpoS has been shown to be an important player in *E. coli*'s osmotic-stress response (22, 61). It activates the transcription of a large number of genes that provide osmoprotection, as well as cross-protection against several other stress factors. The synthesis or uptake of compatible solutes, one of the most important responses of cells against osmotic stress, is partially mediated by RpoS-dependent genes. These include *otsA*, *otsB*, and *treA* (21), involved in trehalose synthesis and metabolism. Nevertheless, the growth of *rpoS* mutants in a hyperosmotic medium, even if slower, was demonstrated and can be attributed to the function of the organic compatible solutes transport systems ProP and ProU, as well as to the high-affinity K^+ transport system KdpFABC and to the BetTIBA system implicated in choline metabolism (9, 63). All of these systems were expressed in an RpoS-independent manner in response to osmotic stress. From our data it is clear that *rpoS*-deficient strains overproduce EPS in response to osmotic stress and that

the deletion of *yjb* or *wca* operons attenuates the already impaired osmotolerance of the mutant.

The CA-EPS has been reported to endow the cells with some stress protection, including against extreme osmotic stress in *E. coli* O157:H7 (6, 34, 35). It has been demonstrated (6) that a CA-EPS-deficient strain lost viability faster than the wild-type strain in the course of a 2-day exposure to 1.5 M or more NaCl. Here we demonstrated that CA-EPS was overproduced in an *rpoS* mutant in response to osmotic stress and that its deficiency limited growth in a saline medium only in an *rpoS*-deficient strain. Many clinical isolates of enteric bacteria were found to be mucoid due to CA-EPS production (20). Furthermore, some *Escherichia coli* O157:H7 isolates were reported to be mucoid only upon growth on medium containing high salt concentrations (26). In view of the increasing reports of the prevalence of mutated *rpoS* genotypes among similar isolates, it may be speculated that these two phenomena, mucosity and loss of *rpoS* functionality, may be coupled, and that the mucoid appearance is a consequence of the *rpoS* mutation.

Several studies have reported varied osmotolerance of clinical *E. coli* isolates (29). It has been shown (9) that an *E. coli* CFT073 lacking *rpoS*, *proP*, and *proU* simultaneously is not limited in growth or virulence in high-osmolality human urine, suggesting that it possesses additional osmoregulatory systems. It will be interesting to determine whether a deletion of *yjb* or *wca* will affect CFT073's growth ability and/or its virulence.

Overproduction of CA-EPS that results in a mucoid colony appearance has been reported to emerge in response to the inactivation of *lon* (36), a treatment that also resulted in UV sensitivity and cell filamentation after UV treatment. Lon is an ATP-dependent protease (4, 7) that, in addition to its role in the degradation of misfolded proteins following heat shock, has a regulatory function (55). Both cell elongation and mucoid colony phenotype were linked to *lon*-specific target proteins RcsA (54) and SulA (37). RcsA is the unstable auxiliary DNA binding protein of the Rcs system (33, 54). SulA is a cell division inhibitor induced as a part of the SOS DNA repair response (47). It binds to FtsZ, a key protein in cell division which is responsible for septum formation (2, 23), and represses cell division (38). Hence, it is likely that in the *yjb rpoS* mutants, both phenotypes are mediated by the Lon protease. We have sequenced the *lon* allele in this double mutant, as well as assayed *lon'*::*lux* activity (not shown), but did not find alteration in sequence or reduced induction.

Our results indicate that there may be a group of genes that are of much greater importance for stress resistance and growth in an *rpoS* mutant than in the wild type. In the present communication, this is demonstrated by the two EPS production operons, *wca* and *yjb*. These two operons are similarly regulated, and their expression is significantly enhanced in the *rpoS* mutant. While the stress resistance capabilities endowed by EPS overproduction are unclear (6), our results clearly show that growth of the mutants impaired in EPS production is inhibited in saline (both *wca* and *yjb*) or even salt-free (*yjb*) medium.

It is tempting to hypothesize that these operons, along with other stress-responsive RpoS-independent systems, have evolved in order to cope with the prevalence of low levels of RpoS or *rpoS* mutations that may have been necessitated by the need for improved nutrient scavenging (14). EPS overproduction may be an example of a mechanism that has evolved to allow cells that have become *rpoS* deficient to grow and cope with osmotic upshifts while enjoying a better nutrient-scavenging capability due to the overexpression of RpoD-dependent uptake and nutrient utilization systems.

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