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The Response Regulator CroR Modulates Expression of the Secreted Stress-Induced SalB Protein in *Enterococcus faecalis*

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The *Enterococcus faecalis* two-component signal transduction system CroRS, also referred as the RR-HK05 pair, is required for intrinsic β-lactam resistance (Y. R. Comenge, R. Quintiliani, Jr., L. Li, L. Dubost, J. P. Brouard, J. E. Hugonnet, and M. Arthur, J. Bacteriol. 185:7184-7192, 2003) and is also suspected to be involved in the expression of *salB* (previously referred to as *sagA*), a gene important for resistance to environmental stress and cell morphology (Y. Le Breton, G. Boël, A. Benachour, H. Prévost, Y. Auffray, and A. Rincé, Environ. Microbiol. 5:329-337, 2003). In this report, we provide genetic and biochemical evidence that *salB* encodes a secreted protein that is expressed from a monocistronic stress-inducible operon. Consistent with CroR being a direct transcriptional activator of the *salB* expression, CroR was found to bind to the *salB* promoter region in electrophoretic mobility shift assays. Interestingly, we provide evidence that SalB does not play a role in the intrinsic β-lactam resistance associated with CroRS. We also show that the CroRS system is able to regulate its own expression. The sequence of the CroRS binding site in the *salB* and *croR* promoter regions was determined using DNase I footprinting assays.

Enterococci have been traditionally considered commensal inhabitants of the gastrointestinal tract of human and animals and able to colonize a large range of ecological niches. Their presence in food products has been regarded as an indicator of insufficient sanitary quality (14), even though they contribute in some cases to flavor development of European dairy products (9). Enterococci were also regarded as low-grade pathogens, involved in few cases of food poisoning, mainly due to production of biogenic amines (7, 14). Over the last two decades, however, they have emerged as significant opportunistic pathogens in intensive care units, particularly affecting immunosuppressed patients (13, 15). *Enterococcus faecalis* is the most common species involved in these enterococcal nosocomial infections. The mechanisms by which this bacterium is able to cross the barrier from inoffensive commensal to major hospital-acquired pathogen are still not well understood. Virulence traits carried principally by mobile genetic elements have been described previously (13, 34), but intrinsic physiological properties of *E. faecalis*, such as its exceptional stress response capacities (29) and inherent antibiotic resistance (21), may also provide an advantage during the infection process as they do for other opportunistic pathogens (39).

The recent availability of the *E. faecalis* V583 genome sequence (28) provides a tool for the identification of potential *E. faecalis* regulatory components, such as alternative sigma factors (2), transcriptional regulators (41, 42), and two-component systems (6, 17, 25, 37), that could be involved in the infection process.

Many aspects of bacterial physiology are under the control of two-component systems (for reviews, see references 18, 19, and 36). These regulatory pathways involve an archetypical mode of signal transduction based on a phosphotransfer from a stress-activated sensor histidine-kinase to its cognate response regulator (35). The response regulator then adjusts gene expression in order for the cell to respond to the signal that initiates the process. Inactivation of two-component system genes in different *E. faecalis* strains (6, 17, 25, 37) failed to uncover a general role for these systems, largely due to heterogeneity within the pool of two-component pathways present in the different *E. faecalis* strains studied. Nevertheless, some *E. faecalis* two-component systems have been shown to be involved in virulence (37), biofilm formation (17), intrinsic antibiotic resistance (6, 17), and/or stress responses (6, 17, 25, 37).

Among the systems analyzed, the CroRS two-component system ("ceftriaxone resistance") (6), previously referred as the RR-HK05 pair by Hancock and Perego (16), appears to be common to several *E. faecalis* strains (16, 25, 37). The CroRS system, composed of the transmembrane CroS sensor histidine-kinase and the OmpR-family CroR response regulator, is essential for intrinsic β-lactam antibiotic resistance (6). The signal recognized by CroS remains unknown, but evidence suggests that it could be related to cell wall perturbations (6). The physiological role of the CroRS system also remains unclear. In a previous study, we noted that a *croR* mutation causes defects in cell morphology and growth (25). These phenotypical features are similar to those we described for a mutation in *salB* (previously referred as *sagA*), a gene whose product is likely involved in cell morphology and stress resistance (23). Additionally, *salB* expression is altered in a *croR*

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mutant, suggesting that the CroRS two-component system could be involved in the regulation of *salB* (25).

In the present study, we found genetic and biochemical evidence that SalB is a stress-activated secreted protein. We then investigated the role of the CroRS two-component system in the regulation of the *salB* gene in *E. faecalis* JH2-2 and showed using electrophoretic mobility shift assays (EMSA) that CroR is able to bind specifically to the *salB* promoter. However, we demonstrated that the intrinsic β-lactam resistance associated with the CroRS system cannot be attributed to the lack of SalB. Additional experiments provided evidence that CroRS autoregulates its own expression, and DNA-binding sites of CroR on these first targets were determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. faecalis* strain JH2-2 (22, 43), a derivative *err05* mutant (affected in *croR*) (25), and a derivative *salB* mutant were used in this study. Cultivation was performed using M17 medium (40) supplemented with 0.5% glucose (GM17) or using brain heart infusion medium at 37°C without shaking. When necessary, erythromycin was added at a concentration of 150 μg ml^{-1}. *Escherichia coli* strains DH5α (32), XL1Blue (Stratagene, La Jolla, CA), and M15(pREP4) (QIAGEN, Valencia, CA) were cultivated under conditions of vigorous agitation at 37°C in LB medium (32) with ampicillin (100 μg ml^{-1}) or kanamycin (25 μg ml^{-1}) when required. MICs of cell wall-active antimicrobials (D-cycloserine, cefotaxime, and ampicillin) were determined with 10^5 CFU per spot on GM17 agar after 48 h of incubation.

Construction of a *salB* mutant by homologous recombination. A 444-bp internal fragment of the *salB* gene was first amplified by PCR using oligonucleotides SalB1 and SalB2 (Fig. 1A; Table 1), digested with SphI and EcoRI, and cloned into these sites in the insertional vector pUCB300. The resulting plasmid, obtained after transformation of *E. coli* XL1Blue, was introduced into *E. faecalis* JH2-2. Integrations by single-crossover recombination within *salB* in erythromycin-resistant colonies were verified by PCR and Southern blot hybridization.

Overexpression and purification of the H_6-CroR protein. A QIAexpress system (QIAGEN, Valencia, CA) was used for the expression of a six-His-tagged CroR recombinant protein (H_6-CroR) as follows. First, the *croR* gene was amplified by PCR using primers CroRN and CroRC (Table 1). The PCR product was then digested using SphI and SalI endonucleases and cloned into the SphI and SalI sites of the pQE-30 plasmid (QIAGEN). The resulting plasmid, pQCroR, was then introduced in *E. coli* M15(pREP4) cells (QIAGEN). Overexpression of the H_6-CroR protein was induced by adding 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to the culture and performing an additional 2-h incubation. Cells were then harvested, washed twice in buffer I (50 mM Tris-HCl, pH 7.5; 50 mM Na_2SO_4; 15% glycerol), resuspended in 5 ml of buffer I, and finally lysed using a cell disrupter (One Shot; Constant Systems, Daventry, England). The H_6-CroR protein was then purified by immobilized metal affinity chromatography from the cell lysate by use of nickel-nitrilotriacetic acid resin (QIAGEN, Valencia, CA), followed by desalting on a PD-10 column (Amersham Biosciences, Piscataway, NJ). Protein concentrations were determined using a method described by Lowry et al. (26).

EMSA. DNA promoter regions were PCR amplified in the presence of 2 μCi [α-^{32}P]dATP. The H_6-CroR protein was diluted in protein buffer (20 mM Tris-HCl, pH 7.5; 2 mM dithiothreitol [DTT]; 50 mM acetyl phosphate). A 10-μl volume of protein was then added to 10 μl of 2× dilution buffer [40 mM Tris-HCl, pH 7.5; 2 mM $CaCl_2$; 2 mM DTT; 20 μg ml^{-1} of poly(dI-dC); 0.2% bovine serum albumin] containing 2.5 ng of DNA. The mixture was incubated for 15 min at room temperature, after which 10 μl of 30% glycerol was added. The products of the reaction were then separated by electrophoresis onto 12.5% polyacrylamide gel and analyzed by autoradiography.

Footprinting experiments. DNase I footprinting assays were performed with a CEQ8000 automated capillary DNA sequencer (Beckman Coulter) using a method based on that previously described by Yindeeyoungyeon and Schell (45). The P*salB* (299 bp) and P*croRS* (358 bp) DNA fragments amplified with primers PsalB1 and PsalB2 and primers Pcro1 and Pcro2, respectively (Table 1), were cloned into in the pGEM-T easy vector by use of a pGEM-T easy vector system (Promega, Madison, WI) as recommended by the supplier. Then, a labeled DNA fragment was obtained by PCR amplification using vector-designed oligonucleotides PU and D4-PR (D4-PR corresponds to the PR oligonucleotide labeled at the 5′ end with Beckman dye D4; Table 1) or oligonucleotides PR and D4-PU and purified using a QIAquick PCR purification kit (QIAGEN). The binding reactions were carried out at room temperature for 5 min in 70-μl reaction volumes containing 33 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 2 mM DTT, 20 μg ml^{-1} poly(dI-dC), 0.02% of bovine serum albumin, 10^3 ng H_6-CroR, 250 ng of labeled DNA fragment, and 1.5 μl of BeF_3^- (a component used to mimic effect of phosphorylation) (44) and generated by mixing NaF and $BeCl_2$ at final concentrations of 0.187 M and 2.27 mM, respectively). The DNase treatment was then performed by addition of 70 μl of a solution containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, and 250 U of DNase I (Amersham Biosciences) and incubation for 1 min at room temperature. The reaction was stopped by addition of 35 μl of 25 mM EDTA and incubation for 5 min at 94°C. DNA was then purified using a QIAquick PCR purification kit (QIAGEN), precipitated by addition of 10% (vol/vol) 100 mM EDTA–10% (vol/vol) 3 M sodium acetate (pH 3.2)–5% (vol/vol) glycogen–3 volumes of ethanol (100%) and resuspended in 40 μl of SLS buffer (Beckman Coulter) before capillary electrophoresis was performed using a CEQ8000 sequencing apparatus (Beckman Coulter). The determination of the DNA sequence of the protected region was performed after comigration of the footprinting assay and the corresponding sequence reaction.

Extraction and separation of secreted proteins. Proteins secreted from *E. faecalis* JH2-2 and mutants *croR* and *salB* were prepared as follows. Cultures were grown to mid-log phase (optical density at 600 nm = 0.5) in 50 ml of brain heart infusion medium. Cells were removed by two rounds of centrifugation for 10 min at 3,220 × *g*. The supernatant was then incubated for 1 h at 37°C with Benzonase (100 U l^{-1}) (Merck) to digest nucleic acids. The supernatant was next mixed with tricholoro-acetic acid to a final concentration of 7% and further incubated at 4°C for 30 min. After a centrifugation at 10,000 × *g* for 10 min, the precipitated proteins were washed with cold acetone and, after an ultimate centrifugation, resuspended in sodium dodecyl sulfate (SDS) gel loading buffer (32). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide).

Mass spectrometry analysis. The peptides of interest were extracted from electrophoresis gel essentially as described by Sauvageot et al. (33) and analyzed with electrospray ionization tandem mass spectrometry. These analyses were carried out with an electrospray ion trap mass spectrometer (LCQ DECA XP; ThermoFinnigan) coupled on line with a high-pressure liquid chromatography apparatus (Surveyor LC; ThermoFinnigan). The peptides were separated by reverse-phase high-pressure liquid chromatography on a C8 capillary column (HyPurity C8) (150 by 0.5 mm), with the parameters set as previously described (33). TurboSEQUEST software was used to compare the amino acid sequences obtained to those of the proteome of *E. faecalis* strain V583 available at the Internet site of The Institute of Genomic Research (http://www.tigr.org).

Transcriptional analysis. Total RNA of *E. faecalis* was isolated using an RNeasy Midi kit (QIAGEN, Valencia, CA). Northern blots of 10 μg RNA normalized by optical density measurement at 260 nm were prepared by using Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ) and standard procedures (32). Dot blots of 2 μg RNA were prepared using a method previously described by Rincé et al. (31) after analysis of RNA with an Experion automated electrophoresis station (Bio-Rad) for normalization and verification of the absence of RNA degradation. Membrane-bound nucleic acids were hybridized to single-strand-labeled probes at 55°C in 1 M sodium phosphate buffer (pH 7.0) containing 5% SDS. After hybridization, membranes were washed twice in 2× SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (10 min) and then twice in 0.5× SSC–0.1% SDS (10 min) at 55°C and exposed to a storage phosphor screen (Packard Instrument Company, Canberra, FL) for 5 h.

A single-strand-labeled probe was obtained as follows: first, a DNA fragment was PCR amplified using *E. faecalis* JH2-2 chromosomal DNA as a template and primer pairs SalB1 and SalB2 (461 bp) (Table 1). The probe was then synthesized by elongating one oligonucleotide (SalB2) with *Taq* DNA polymerase, 2 μM concentrations of dCTP, dGTP, and dTTP, 2 μCi of [α-^{32}P]dATP, and 10 ng of the previously obtained PCR DNA fragment as a template. Thirty cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C were performed.

The 5′ end of *salB* mRNA was mapped from a 5′-RACE (5′-rapid amplification of cDNA ends) PCR product obtained with a 3′/5′-RACE kit (Roche Molecular Biochemicals) using primer SalBRace for cDNA synthesis from total RNA and PsalB2 for DNA sequencing.

General methods. Restriction endonucleases, alkaline phosphatase, and T4 DNA ligase were obtained from Amersham International, Promega (Promega, Madison, WI), and Roche Applied Science (Roche, Indianapolis, IN) and used according to the manufacturers' instructions. PCR was carried out in a reaction volume of 25 μl using 5 ng of chromosomal DNA of *E. faecalis* strains and *Taq* DNA polymerase from Amersham International. The annealing temperature was 5°C below the melting temperature of the primers; 30 cycles were performed, and PCR products were purified using a QIAquick PCR

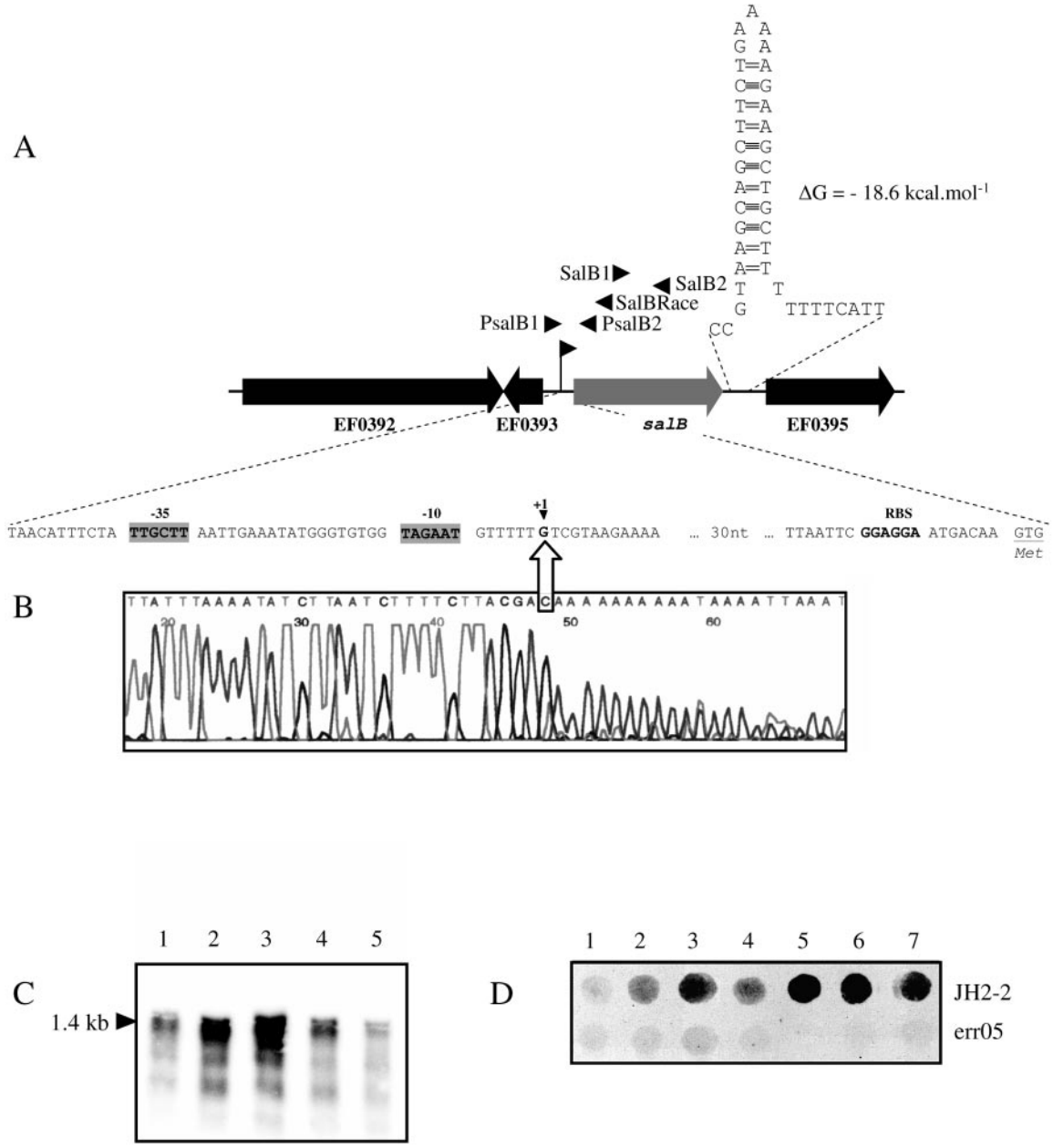

FIG. 1. (A) Schematic representation of the genetic organization of the *salB* chromosomal region. Large arrows represent the ORFs, and their orientation shows the transcriptional direction. The nucleotide sequences of the *salB* promoter region and of a putative Rho-independent terminator located 24 nucleotides downstream of the *salB* stop codon are shown. The transcriptional initiation nucleotide (+1), the putative −35 and −10 motifs, and the putative ribosome binding site sequence are indicated. (B) Electropherogram obtained from 5′-RACE PCR experiments. The sequence in the electropherogram was obtained using the primer PsalB2, 5′ A-tailed cDNA prepared from total RNA, a 3′/5′ RACE kit (Roche Applied Sciences), and primer SalBRace. The last base upstream of the A tail corresponded to the first nucleotide transcribed. The corresponding G on the reverse-complement strand is indicated (+ 1). (C) Analysis of *salB* expression. The results of Northern blot hybridization of *E. faecalis* JH2-2 RNA extracted from exponentially growing cells (lane 1), from cells incubated for 10 min with 0.3 M NaCl (lane 2), 10 min at 50°C (lane 3), or 10 min in GM17 medium adjusted to pH 4.8 with lactic acid (lane 4), or from cells harvested at the entrance to stationary phase (lane 5) are shown. Hybridization was performed with a single-strand DNA probe corresponding to the DNA region located between primers SalB1 and SalB2. The size of the transcript was estimated by comparison with an RNA ladder (Amersham International) (0.56 to 9.4 kb). (D) Dot blot hybridization of total RNA from *E. faecalis* cells of strain JH2-2 and of the *err05* mutant strain harvested in the exponential-growth phase (1) and from exponentially growing cells incubated in the presence of 0.3 M NaCl for 5, 10, and 20 min (lanes 2, 3, and 4, respectively) or at 50°C for 5, 10, and 20 min (lanes 5, 6, and 7, respectively). Hybridization was performed with the same probe as described for panel C.

purification kit (QIAGEN). *E. coli* and *E. faecalis* were transformed using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) as described by Dower et al. (8) and Holo and Nes (20), respectively. Plasmids were purified using a QIAprep Miniprep kit (QIAGEN). DNA and amino acid sequences were analyzed using Mac Vector software (Kodak; Scientific Imaging Systems, New Haven, CT), and databases searches were performed with the BLAST program (1). Other standard techniques were carried out as described by Sambrook et al. (32).

TABLE 1. Primers used for PCR, 5′-RACE PCR, and EMSA experiments

Primer	5′–3′ sequence[a]	Position	Direction[b]
SalB1	gggcAtgcAAAGAAGACAAACAAGCC (*SphI*)	*salB* ORF	+
SalB2	ccgaAttcGCCAGTATTTTCTGTTGC (*EcoRI*)	*salB* ORF	−
SalBRace	TTTTATCAACATTGTCCGCC	*salB* ORF	−
PsalB1	AAAGAAGCAAAATTTGAATAAAAA	*salB* promoter region	+
PsalB2	CTGATGCAAATAGACGTTTC	*salB* ORF	−
Pcro1	GCGACATGTAATAATATACC	*croR* promoter region	+
Pcro2	GCTTTCACGACTTCGTATCC	*croR* ORF	−
CroRN	acagcatgcAAAATTTTAGTTGCAGATGATG (*SphI*)	*croR* ORF	+
CroRC	acagTcGacTTAACGAGCATCGATCTTAT (*SalI*)	*croR* ORF	−
PU	GTAAAACGACGGCCAGT	PGEM-T easy vector	
PR	CAGGAAACAGCTATGAC	PGEM-T easy vector	

[a] Bases in lowercase letters are not complementary to the target sequence. Underlined bases correspond to restriction sites (given in parentheses).
[b] +, primer directed towards the 3′ end of the genes; −, primer in the opposite direction.

RESULTS

Genetic organization of the *salB* locus. We previously identified the *salB* gene during screening for stress response mutations following random mutagenesis of *E. faecalis* JH2-2 (23). The *salB* mutant displayed enhanced sensitivity toward different stress challenges (sensitivity to NaCl, heat shock, SDS, ethanol, H_2O_2, alkaline, or acid pH) as well as altered cell shape and septation anomalies.

The 1,350-nucleotide *salB* sequence is preceded by two open reading frames (ORFs), EF0392 and EF0393, encoding proteins of unknown function; the EF0395 gene, located downstream of *salB*, encodes a 42.2-kDa protein homologous to methionine synthetase (Fig. 1A). To determine the size of the *salB* transcription unit, Northern blot hybridizations were performed using total RNA extracted from exponentially grown *E. faecalis* strain JH2-2 and a DNA probe complementary to the

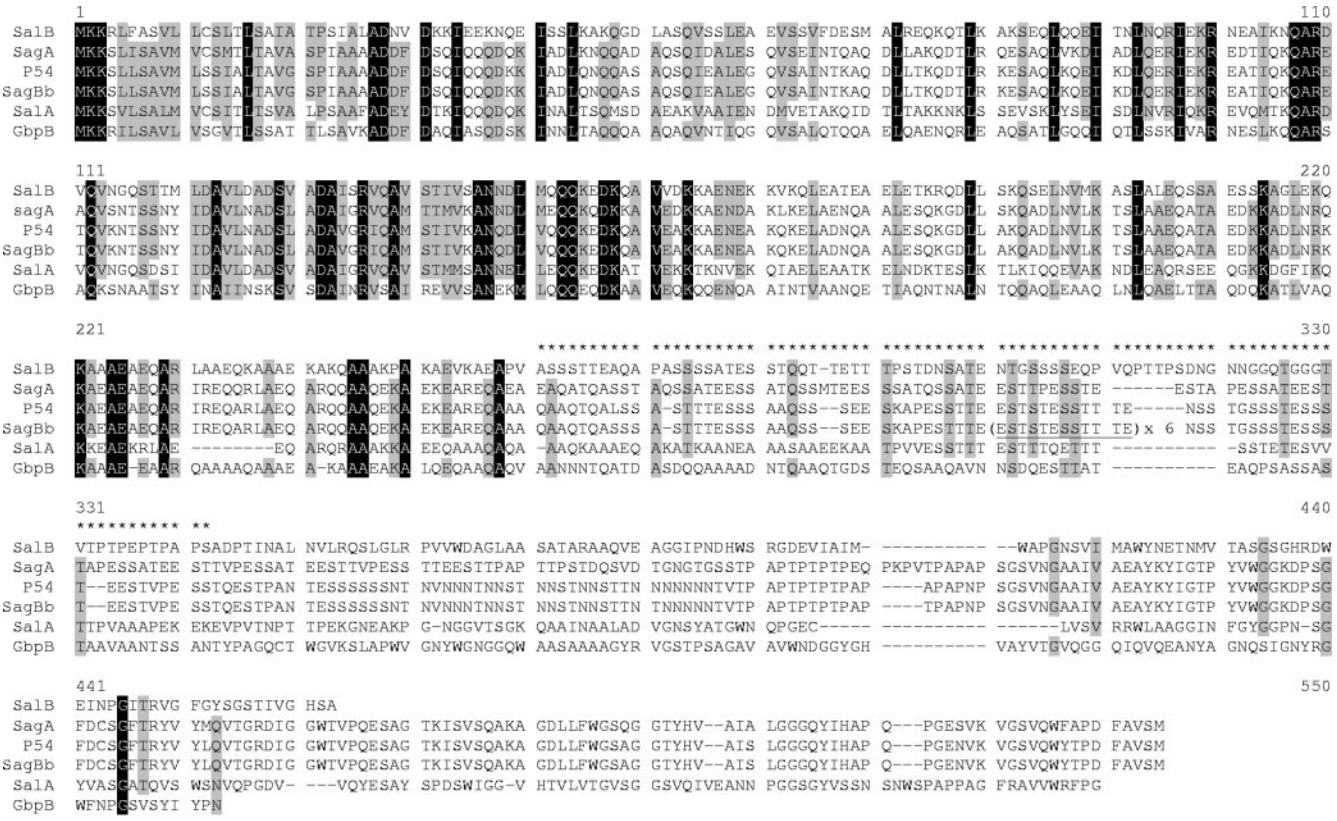

FIG. 2. Amino acid sequence alignment of SalB with SagA and P54 from *Enterococcus faecium* (10, 38), SagBb from *Enterococcus hirae* (F. Teng, B. E. Murray, and G. M. Weinstock, unpublished data), SalA from *E. faecalis* (28), and GbpB from *Streptococcus mutans* (4, 5, 27). Residues indicated with gray characters on a black background are identical within all proteins. Functionally related amino acids (H, K, and R; F, Y, and W; L, I, M, and V; G and A; S and T; D and E; N and Q; C and P) found at the same position in at least five proteins are indicated with black characters on a gray background. Asterisks show the SalB central domain of unusual composition. Underlined amino acids in SagBb represent a 12-amino-acid motif present once in P54 and six times in SagBb.

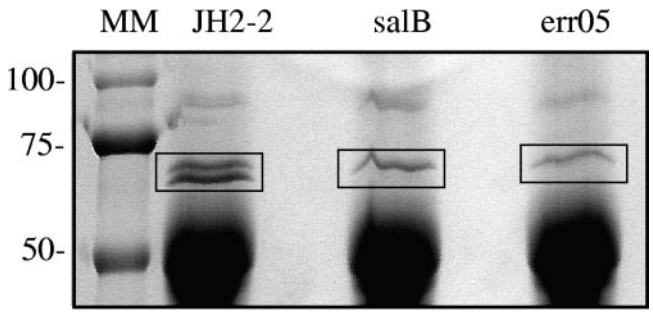

FIG. 3. Separation by SDS-PAGE of secreted proteins extracted from strain JH2-2 and the *salB* and *err05* mutant strains. MM, molecular markers. Numbers at the left indicate the molecular masses of the markers in kilodaltons. Proteins contained in the framed areas of the electrophoresis gel were digested with trypsin and identified by comparison with proteins deduced from the *E. faecalis* V583 sequenced genome by use of TurboSEQUEST software (see Table 2).

salB gene (Fig. 1C). A 1.4-kb unique transcript, similar in size to the *salB* ORF itself, was detected in the assay, suggesting that the *salB* gene is transcribed independently of the surrounding genes. Consistent with this result, we also identified 24 nucleotides downstream of the *salB* stop codon (TAA), an invert-repeat sequence that likely corresponds to a Rho-independent terminator ($\Delta G = -18.6$ kcal mol^{-1}) (Fig. 1A). RNA extracted from exponentially growing *E. faecalis* JH2-2 cells was then used to map the *salB* transcriptional start site by 5′-RACE PCR. The resulting electropherogram presented in Fig. 1B localizes the transcriptional start point (+1) of the *salB* gene at a site 49 nucleotides upstream of a potential ribosome-binding site (GGAGGA). Seven base pairs upstream of the transcriptional start site lies a σ^A-like promoter element with a putative −10 box (TAGAAT) separated by 19 nucleotides from a putative −35 box (TTGCTT) (Fig. 1A). These results strongly suggest that *salB* belongs to a monocistronic operon that is transcribed in mid-exponential phase from a promoter recognized by RNA polymerase carrying the housekeeping sigma factor σ^A.

Previous data showed that a *salB* mutant was more sensitive to several stress conditions, including heat shock and hyperosmotic stress (23). This prompted us to test whether *salB* expression could be influenced by stress. Northern blot hybridization of total RNA extracted from *E. faecalis* cells exposed for 10 min to different culture conditions (the presence of NaCl, 50°C, acid pH) demonstrated that the level of *salB* mRNA increases when *E. faecalis* cells are subjected to an osmotic stress or a high temperature challenge (Fig. 1C). The stress inducibility of *salB* is consistent with the anticipated role of its product as a protein that has a protective function needed when the cell encounters detrimental environmental situations. The effect of the inactivation of *croR* on the induction of *salB* was tested by RNA hybridization (Fig. 1D). Dot blot hybridization of total RNA extracted from *E. faecalis* cells exposed for 5, 10, or 20 min to 0.3 M NaCl or to 50°C showed that CroR is required for *salB* induction.

***salB* encodes a secreted protein.** The *salB* sequence encodes a protein of 449 amino acids (aa) (Fig. 2) with a calculated molecular mass of 47.3 kDa and an estimated pI of 4.7. The first 27 amino acids contain the elements found in gram-positive signal peptides. Cleavage after residue 27 by a signal peptidase would result in a secreted SalB protein of 44.5 kDa. Homology searches revealed that the N-terminal portion of SalB (from aa 1 to aa 260) is similar to the N-terminal portions of the proteins SagA (also referred as P60) and P54 from *Enterococcus faecium* (10, 38) and SagBb from *Enterococcus hirae* (F. Teng, B. E. Murray, and G. M. Weinstock, unpublished data) and the SalA antigen (putative secreted lipase) from *E. faecalis* (28). SalB also shares homology with the N-terminal portion of PcsB-like proteins from different species of streptococci such as *Streptococcus mutans* GbpB (4, 5, 27) (Fig. 2). Interestingly, SagA, P54, GbpB, and a number of "PcsB-like" proteins have been shown to be associated with the cell wall or secreted into the growth medium. At least some of these proteins appear to be involved in cell wall hydrolysis and in the regulation of cell wall biosynthesis (4, 10, 27, 38). The central domain of these proteins, as with that of SalB (aa 261 to aa 341), presents an unusual composition: 46 amino acids out of 81 of this SalB segment are serine, threonine, or proline. In its C-terminal portion, SalB shares significant homology with proteins deduced from the *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 genome (accession no. ZP00063637 and ZP00063635) containing Lys7 domains, which are usually found in proteins that bind to cell wall peptidoglycan (3).

We extracted and separated through SDS-PAGE the secreted proteins from the *E. faecalis* JH2-2 wild-type strain and from the *salB* and *err05* mutant strains (Fig. 3). Results showed that the *salB* and *err05* mutants were missing a protein that migrated between 50- and 75-kDa molecular mass markers. Mass spectrometry analyses revealed that the missing protein corresponds to SalB (Table 2). The other protein closely migrating with SalB was also identified as the putative secreted lipase SalA (28).

Our results indicate that SalB is a secreted protein. Addition-

TABLE 2. Identification of secreted proteins by differential analysis using mass spectrometry[a]

Strain	Protein identified	Molecular mass (kDa)	pI	Protein length (aa)	Score[b]	Protein coverage (%)		No. of peptides sequenced
						By aa count	By mass	
JH2-2	SalA	50.9	4.8	482	110	24.9	24.5	11
	SalB	47.3	4.7	449	88	18.9	19.5	9
salB mutant	SalA	50.9	4.8	482	80	19.3	18.9	8
err05 mutant	SalA	50.9	4.8	482	90	19.7	20.2	9

[a] Proteins contained in the framed areas of the electrophoresis gel presented in Fig. 3 were digested with trypsin and identified by comparison with proteins deduced from the *E. faecalis* V583 sequenced genome by use of the program TurboSEQUEST.
[b] Score given by the TurboSEQUEST program.

TABLE 3. Susceptibility of *E. faecalis* strains to cell wall-active antimicrobials

Strain	MIC (μg ml^{-1}) of:		
	D-Cycloserine	Cefotaxime	Ampicillin
JH2-2	250	500	1
err05 mutant	50	0.25	0.2
salB mutant	250	500	1

ally, the absence of SalB in the media of the strain with the *croR* mutation is consistent with the notion that the CroRS two-component system is involved in *salB* expression or secretion.

As previously shown by Comenge et al. (6), a *croR* mutation led to a decrease in the MICs of cell wall-active antimicrobials (Table 3). In order to investigate whether this phenotype could be attributed to a CroR effect on the SalB protein, we tested the susceptibility of the *salB* mutant to the cell wall-active antimicrobials. As presented in Table 3, similar resistance levels were observed for the *salB* mutant and the wild-type strain, showing that the antibiotic sensitivity of the *err05* mutant cannot be attributed to the nonexpression of the SalB protein.

CroR binds to the *salB* promoter region. To test whether the CroR response regulator could bind directly to the *salB* promoter region, we purified a CroR protein harboring an N-terminal six-histidine tag (H_6-CroR) as described in Materials and Methods. The purified protein was visualized on a Coomassie-stained SDS-PAGE gel (Fig. 4A) and was shown to migrate in accordance with its estimated molecular size of 27 kDa.

A 299-bp DNA fragment containing the promoter region of *salB* (P*salB*) (nucleotide −212 to nucleotide +87 relative to the transcriptional start point) was PCR amplified with primers PsalB1 and PsalB2 (Table 1) and used as a DNA target for EMSA. As shown in Fig. 4B, when the P*salB* fragment is incubated with different amounts of H_6-CroR in the presence of 50 mM acetyl phosphate, three bands with reduced mobility, designated complex 1 (C1), complex 2 (C2), and complex 3 (C3), were observed. Decreases in the amount of H_6-CroR in the binding assay mixture progressively led to a reduction in the amounts of C1, C2, and C3 and a concomitant increase in the amount of free unbound target DNA (Fig. 4B). The binding of H_6-CroR to the *salB* promoter region appeared to be specific in that the addition of unlabeled 299-bp P*salB* DNA reduced the abundance of the putative complexes whereas addition of a nonspecific competitor (an unlabeled internal fragment of the *salB* ORF) did not (Fig. 4B). In keeping with the expectation that it is the phosphorylated form of CroR that is the DNA-binding species, no H_6-CroR-dependent retardation of P*salB* was observed when the EMSA was performed in the absence of the phosphate donor acetyl-phosphate (data not shown).

DNase I footprinting experiments were then performed in order to determine the sequence of the CroRS binding site in the P*salB* DNA fragment. As shown in Fig. 5, H_6-CroR protects a 45-bp region extending from positions −1 to +44 relative to the transcription initiation site.

The results of these experiments, taken together with those showing the *croR* dependency of the SalB expression, support the idea of a direct involvement of the CroRS two-component system in the activation of *salB*.

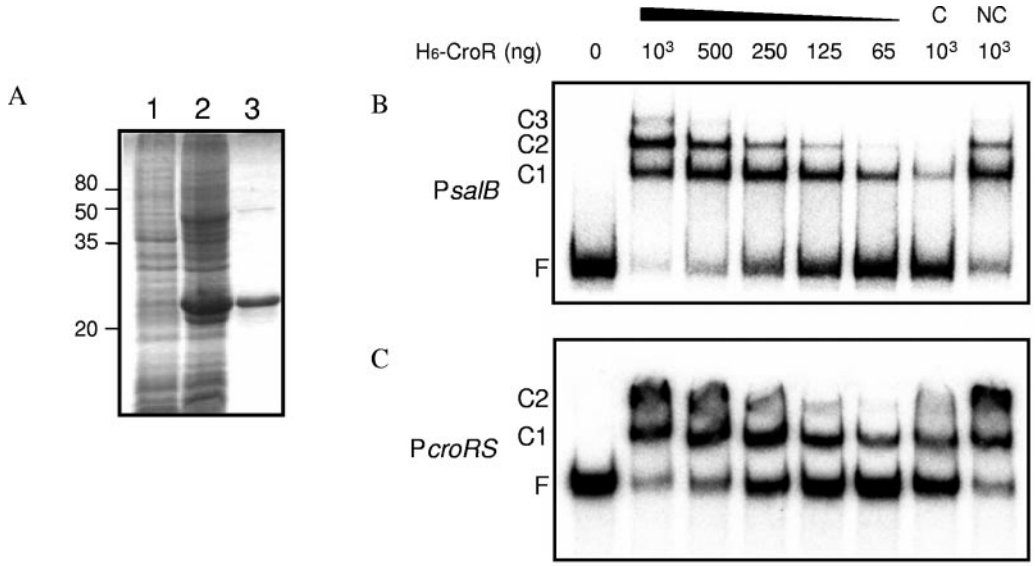

FIG. 4. (A) Purification of the recombinant protein H_6-CroR. The results of Coomassie brilliant blue-stained SDS-PAGE of *E. coli* extracts before and after IPTG induction (lanes 1 and 2, respectively) and of purified H_6-CroR protein (lane 3) are shown. Numbers on the left indicate molecular masses of the markers in kilodaltons. (B and C) EMSA of the promoter regions of the *salB* gene and of that of the *croRS* operon with H_6-CroR protein. EMSA were performed with 2.5 ng of labeled DNA, corresponding to the promoter region of the *salB* gene (P*salB*; 299 bp) (B) or of the *croRS* operon (P*croRS*; 358 bp) (C) and different amounts (indicated above) of the purified H_6-CroR protein incubated in the presence of 50 mM acetyl phosphate. To test the specificity of H_6-CroR binding, a 50× excess of unlabeled competitor DNA (lane C, corresponding to P*salB* in panel B or to P*croRS* in panel C) or of unlabeled no-competitor DNA (lane NC, corresponding to an internal segment of *salB*) was added in the binding mixture. The positions of the free DNA (F) and the H_6-CroR/DNA (C1, C2, and C3) complexes are indicated on the left.

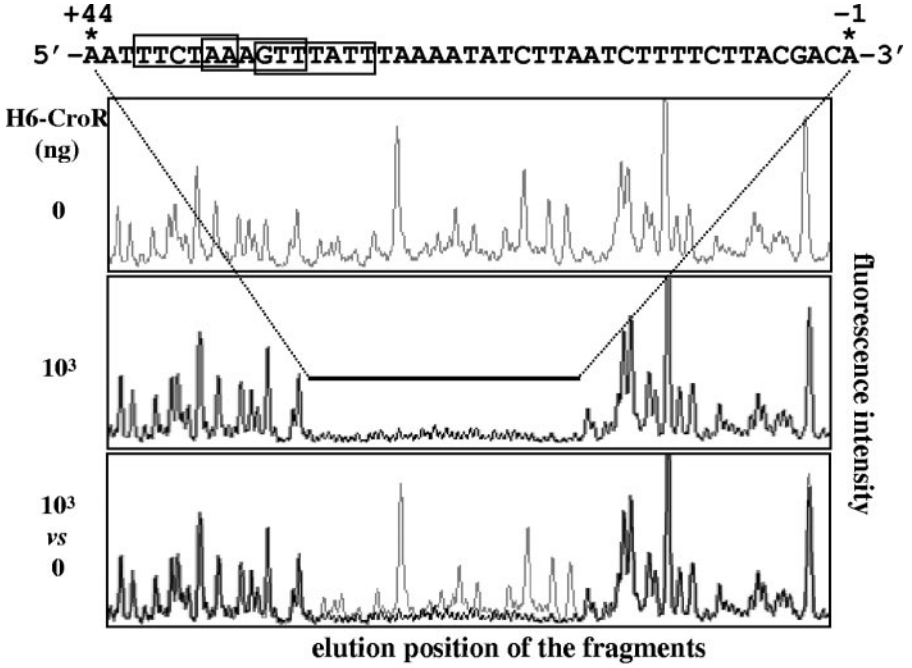

FIG. 5. DNase I footprinting assay of H_6-CroR with a D4-labeled DNA fragment containing the P*salB* promoter region. Amplification was performed with PU and D4-PR. Electropherograms of reactions performed with or without H_6-CroR give fluorescence intensity (fragment abundance) in the *y* axis and elution position of the fragments (size) in the *x* axis. The solid line indicates the DNA region protected from DNase I by H_6-CroR. The corresponding DNA sequence determined as described in Materials and Methods is indicated. The three 6- to 7-bp motifs (TTCTAAA, AAAGTT, and GTTTATT) conserved in the DNA sequence protected by H_6-CroR on the P*salB* and the P*croR* promoter regions are boxed.

The CroRS system regulates its own expression. Autoregulation is a common feature for two-component systems. Comenge et al. (6) suggested that this could also be true for CroRS. The *croRS* locus is expressed as a bicistronic operon from a unique promoter located upstream of the *croR* gene (6, 25). A 358-bp DNA fragment containing the promoter region of *croRS* (P*croRS*; nucleotide −133 to nucleotide +125 relative to the transcription initiation site determined by Comenge et al.) (6) was obtained by PCR amplification using the primers Pcro1 and Pcro2 (Table 1) and used for EMSA with different amounts of phosphorylated H_6-CroR. As was the case with the DNA fragment containing the *salB* promoter, slower migrating bands (C1 and C2) were observed whose abundance was specifically reduced by the addition of unlabeled P*croRS* DNA (Fig. 4C). The apparent binding of CroR-PO_4 to the *croR* promoter is consistent with autoregulation of the *croRS* operon.

The DNA sequence of the CroRS binding site in the P*croRS* DNA fragment was determined using DNase I footprinting experiments and both DNA strands (Fig. 6). Results revealed a 45-bp common protected region corresponding to positions −87 to −43 relative to the transcription initiation site. Comparison of this DNA sequence to that protected in the *salB* promoter region revealed three conserved 6- to 7-bp motifs (TTCTAAA, AAAGTT, and GTTTATT) which could be candidates for a CroR recognition sequence.

DISCUSSION

Over the last several years, we investigated the *E. faecalis* environmental stress response by use of two-dimensional electrophoresis (for reviews, see references 12 and 29) and genetic analyses of *E. faecalis* strain JH2-2 (11, 23–25, 29–31). The genetic approach allowed us to identify two independent mutants presenting similar growth defects and altered cell morphology (23, 25). Genotypic analysis revealed that one mutation (BS9) lies in the *salB* gene (previously referred as *sagA*) (23), encoding a putative secreted protein with unknown function (23). The second mutation mapped to a gene encoding an OmpR-like response regulator (25), recently redesignated CroR (6). Northern blot hybridization experiments revealed impaired *salB* expression in the *err05* mutant, suggesting a possible involvement of the CroR regulator in the control of *salB* expression (25).

In the present report, the *salB* gene is shown to be transcribed as a 1.4-kb monocistronic mRNA, the expression of which is induced by environmental stresses such as high osmolarity and temperature. Interestingly, dot blot hybridization experiments also revealed that when the *err05* mutant strain is subjected to these two stress conditions, the *salB* expression is not activated. This result strongly suggests that CroR might be required for *salB* expression when *E. faecalis* cells are subjected to high osmolarity and temperature.

We then tested the hypothesis of the direct involvement of CroR in *salB* regulation, and EMSA experiments gave evidence that the phosphorylated CroR response regulator is able to bind specifically to the *salB* promoter region. This result, in conjunction to the previous demonstration of the reduced *salB* expression in the *err05* mutant strain lacking *croR* (25), argues that CroR is likely to be a transcriptional regulator involved in

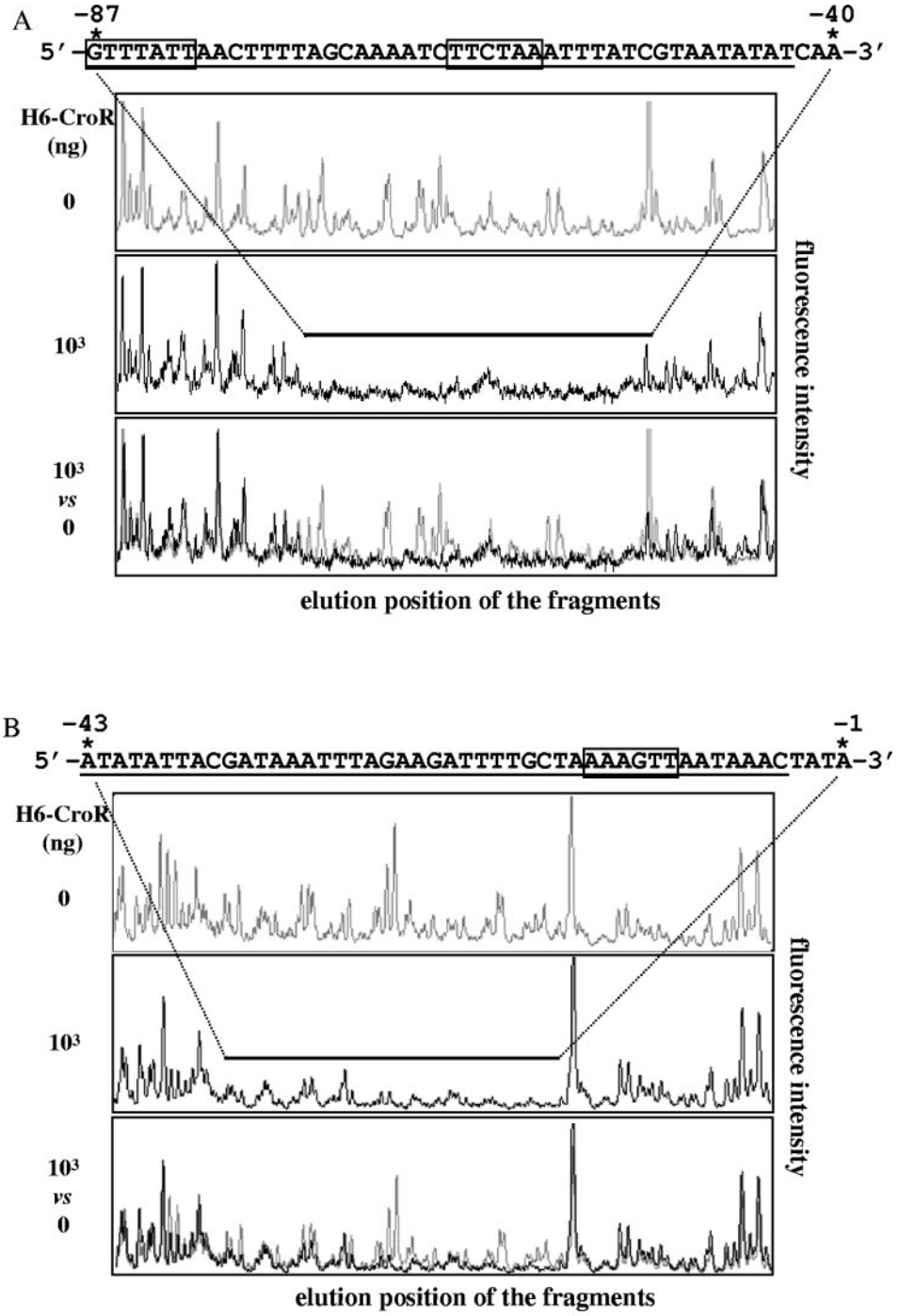

FIG. 6. DNase I footprinting assays of H_6-CroR with D4-labeled DNA fragments containing the P*croRS* promoter region. Amplifications were performed with PU and D4-PR (A) and PR and D4-PU (B). Electropherograms of reactions performed with or without H_6-CroR that indicate fluorescence intensity (fragment abundance) in the *y* axis and elution position of the fragments (size) in the *x* axis are shown. The solid lines indicate DNA regions protected from DNase I by H_6-CroR. The DNA sequence of the protected region is indicated, and underlined nucleotides represent the common protected region between both experiments. The three 6- to 7-bp motifs (TTCTAAA, AAAGTT, and GTTTATT) conserved in the DNA sequence protected by H_6-CroR on the P*salB* and the P*croR* promoter regions are boxed.

salB activation in *E. faecalis* JH2-2. Given that CroR can be phosphoactivated by its mated histidine-kinase CroS (6), we can conclude that the CroRS two-component system is involved in the *salB* regulation. To our knowledge, this result corresponds to the first demonstration of the presence in *E. faecalis* of a signal transduction pathway involved in the direct regulation of a stress protein.

Inactivation of *salB* or *croR* genes confers growth defects and alterations of cell morphology (23, 25), so we can suggest that these phenotypes in the *err05* mutant strain may be the

consequence of the low level of SalB. The first role for the CroRS system to be described was its involvement in *E. faecalis* intrinsic β-lactam resistance (6), and it was tempting to see whether SalB could actually be involved in β-lactam resistance. The sensitivity to cell wall-active antimicrobials was tested, and the results showed that the decreases of the MICs of D-cycloserine, cefotaxime, and ampicillin were observed only in the *err05* mutant strain but not in the *salB* mutant strain, demonstrating that SalB is not involved in the CroRS-associated β-lactam resistance.

A previous work showed that a mutation in the *E. faecalis salB* locus led to cell shape anomalies, nonsymmetrical divisions, and sensitivity to different environmental stresses (23). Here, we show that SalB is a secreted protein with homology to PcsB-like proteins involved in cell wall hydrolysis and the regulation of cell wall biosynthesis. SalB shares homologies with the *E. faecalis* SalA protein. The latter is also a PcsB-like protein, and chromosomal location of *salA* is similar to that observed for other *pcsB*-like genes, as it is located downstream of the *mreCD* locus, encoding proteins known to play a role in cell shape. In our experiments, we demonstrated that SalA is also a secreted protein. However, in contrast to the results seen with *salB*, *salA* seems not to be regulated by the CroRS two-component system.

EMSA analyses also showed that CroR binds to the promoter region of the *croRS* locus. This result demonstrates the validity of the hypothesis, based on analyses of the activity of a P*croRS-lacZ* fusion in *croRS* mutants (6), that the CroRS two-component system is able to regulate its own expression.

DNase I footprinting experiments allowed determination of the sequences of 45-bp DNA segments protected from DNase I by H_6-CroR binding in the promoter region of genes *croR* and *salB*. Both protected sequences contain low (<18%) G+C content, and this is also the case for the three 6- to 7-bp motifs common in both protected regions. Thus, we can expect a high occurrence of the CroR recognition sequence in the AT-rich *E. faecalis* genome and consequently a large number of genes controlled by the two-component system CroRS.

The sensitivity of the *err05* mutant strain lacking *croR* to the cell wall-active antimicrobials cannot be explained solely by the low level of SalB in this strain, and additional target(s) might correspond to genes involved in this phenotype. Experiments are now in progress in our laboratory to identify new genes regulated by CroRS.

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