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The CroRS Two-Component Regulatory System Is Required for Intrinsic β-Lactam Resistance in *Enterococcus faecalis*

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Enterococcus faecalis **produces a specific penicillin-binding protein (PBP5) that mediates high-level resistance to the cephalosporin class of β-lactam antibiotics. Deletion of a locus encoding a previously uncharacterized two-component regulatory system of *E. faecalis* (*croRS*) led to a 4,000-fold reduction in the MIC of the expanded-spectrum cephalosporin ceftriaxone. The cytoplasmic domain of the sensor kinase (CroS) was purified and shown to catalyze ATP-dependent autophosphorylation followed by transfer of the phosphate to the mated response regulator (CroR). The *croR* and *croS* genes were cotranscribed from a promoter (*croRp*) located in the *rrnC-croR* intergenic region. A putative seryl-tRNA synthetase gene (*serS*) located immediately downstream from *croS* did not appear to be a target of CroRS regulation or to play a role in ceftriaxone resistance. A plasmid-borne *croRp-lacZ* fusion was *trans*-activated by the CroRS system in response to the presence of ceftriaxone in the culture medium. The fusion was also induced by representatives of other classes of β-lactam antibiotics and by inhibitors of early and late steps of peptidoglycan synthesis. The *croRS* null mutant produced PBP5, and expression of an additional copy of *pbp5* under the control of a heterologous promoter did not restore ceftriaxone resistance. Deletion of *croRS* was not associated with any defect in the synthesis of the nucleotide precursor UDP-MurNAc-pentapeptide or of the D-Ala$_4$→L-Ala-L-Ala-Lys$_3$ peptidoglycan cross-bridge. Thus, the *croRS* mutant was susceptible to ceftriaxone despite the production of PBP5 and the synthesis of wild-type peptidoglycan precursors. These observations constitute the first description of regulatory genes essential for PBP5-mediated β-lactam resistance in enterococci.**

Enterococcus faecalis and *E. faecium* are opportunistic pathogens that are common causes of urinary tract infections, bacteremia, and endocarditis (20). Enterococcal infections are difficult to treat, as enterococci are intrinsically resistant to various antibiotics and can acquire, mainly by horizontal gene transfer, high-level resistance to virtually all antimicrobial agents. The complete genome sequence of *E. faecalis* strain V583 revealed an unusually high (25%) content of mobile elements and exogenously acquired DNA, including virulence factors and antibiotic resistance genes (22). The plasticity of the genome correlates with the facility of enterococci to acquire novel resistance mechanisms and to transfer the corresponding genes to other genera, as exemplified by the emergence of high-level glycopeptide resistance in *E. faecalis* and *E. faecium* in the late 1980s and the dissemination of the same gene cluster in *Staphylococcus aureus* 15 years later (8).

Enterococci are resistant to the newer cephalosporins which have been developed to treat infections due to gram-negative bacteria producing β-lactamases. Treatment with cephalosporins is one of the risk factors for colonization and infection by multidrug-resistant enterococci. Cephalosporin resistance is mediated by a specific class B penicillin-binding protein (PBP) commonly referred to as low-affinity PBP5 (7, 27). Production of PBP5 also confers moderate-level resistance to ampicillin (MIC, 2 to 16 μg/ml). Acquisition of higher levels of ampicillin resistance, seen mainly in *E. faecium*, results from overproduction of PBP5 (11, 14), amino acid substitutions that further decrease interaction of PBP5 with β-lactams (26, 32), and modification of as-yet-unidentified non-PBP factors (18, 29).

In this report, we show that a two-component regulatory system (designated CroRS [for "ceftriaxone resistance"]) is essential for intrinsic β-lactam resistance in *E. faecalis*. This system, designated RR05-HK05 in the classification of Hancock and Perego (15), was initially chosen because of sequence similarity with two-component systems that control acquired enterococcal resistance to the glycopeptide antibiotics vancomycin and teicoplanin (2, 15). We report the resistance phenotype associated with deletions from the *croRS* locus, purification of the CroR response regulator and of a soluble fragment of the CroS histidine protein kinase to test their activity, and transcriptional analysis of the *croRS* locus. Since defects in the assembly of peptidoglycan precursors are associated with impaired expression of methicillin resistance in *S. aureus* (10, 25), we also analyzed the impact of the *croRS* deletion on the assembly of cytoplasmic precursors and on peptidoglycan cross-bridge formation.

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MATERIALS AND METHODS

Growth conditions and susceptibility tests. Bacterial strains were grown in brain heart infusion (BHI) broth or agar (Becton Dickinson, le Pont de Claix,

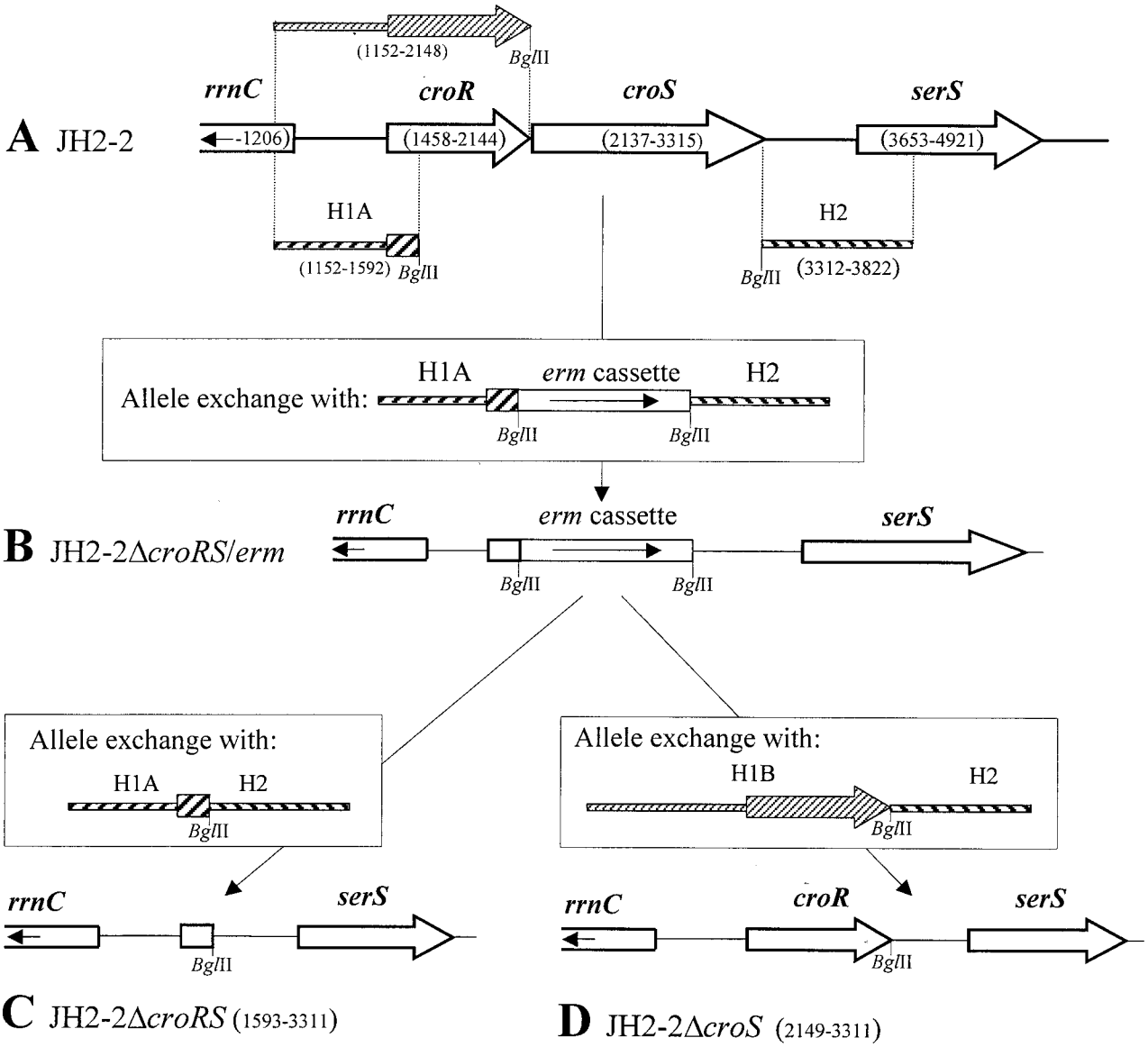


FIG. 1. Deletions from the *croRS* locus. (A) Map of the wild-type locus of *E. faecalis* JH2-2 and location of the DNA fragments (H1A, H1B, and H2) used for allele exchange by homologous recombination. Numbers in parentheses indicate the coordinates of the extremities of the *croR*, *croS*, and *serS* open reading frames (open arrows), of the H1A, H1B, and H2 DNA fragments (hatched), and of a portion of the *rrnC* rRNA gene cluster (open box). *Bgl*II restriction sites were introduced at one extremity of H1A, H1B, and H2. (B) Replacement of *croRS* by an erythromycin resistance gene cassette (*erm*). JH2-2Δ*croRS*/*erm* was obtained by homologous recombination between the wild-type *croRS* locus of JH2-2 and a derivative of the thermosensitive plasmid pHS1 carrying the H1A-*erm*-H2 DNA insert depicted in the inset. (C) To construct JH2-2Δ*croRS*, the *erm* cassette was deleted from the chromosome of JH2-2Δ*croRS*/*erm* by allele exchange with H1A directly linked to H2. (D) To construct JH2-2Δ*croS*, the *erm* cassette of JH2-2Δ*croRS*/*erm* was replaced (using H1B linked to H2) by the *croR* open reading frame. Numbers in parentheses indicate the extremities of the deletions from JH2-2Δ*croRS* and from JH2-2Δ*croS*. Coordinate 1 corresponds to position 3,169,253 of the assembled *E. faecalis* genome at www.tigr.org.

France) at 37°C. MICs of ampicillin (Bristol-Myers, Paris, France) and ceftriaxone (Laboratoires Roche, Neuilly, France) were determined with 10^5 CFU per spot on BHI agar after 48 h of incubation.

Deletion of the *croR* and *croS* genes. Deletions were made from the chromosome of *E. faecalis* JH2-2 by homologous recombination using derivatives of the suicide vector pHS1, which is thermosensitive for replication and confers gentamicin resistance (A. Arbeloa and M. Arthur, unpublished data). Briefly, DNA fragments (H1A, H1B, and H2) flanking the sequences targeted for deletion were amplified with primers (for H1A, primers 5′-ATTGATTTCTGAATCGC-3′ and 5′-AGATCTATCTGGTGTTGTGTGC-3′; for H1B, primers 5′-ATTGATTTCTGAATCGC-3′ and 5′-TTTTAGATCTTTAACGAGCATCGATCTTAT-3′; and for H2, primers 5′-AGATCTGAGTTAATTGACATCCC-3′ and 5′-GCAGACACATCATTCCG-3′) containing *Bgl*II restriction sites (underlined) to facilitate subsequent cloning steps. The fragments were cloned (with or without an intervening *Bgl*II erythromycin resistance cassette [*erm*]) into pHS1 to generate the inserts (H1A-*erm*-H2, H1A-H2, and H1B-H2) as shown in the insets in Fig. 1. To replace *croRS* by *erm* (Δ*croRS*/*erm*), the derivative of plasmid pHS1 carrying H1A-*erm*-H2 (pHS1ΩH1A-*erm*-H2) was introduced by electrotransformation into *E. faecalis* JH2-2. Replacement of *croRS* by *erm* was selected on agar containing erythromycin (10 μg/ml) at the nonpermissive temperature (42°C) for plasmid replication (Fig. 1B). One clone resistant to erythromycin and susceptible to gentamicin was identified by replica plating on BHI agar containing gentamicin (128 μg/ml) and was designated JH2-2Δ*croRS*/*erm*.

Deletion of the *erm* cassette of JH2-2Δ*croRS*/*erm* was obtained in two steps

using a derivative of pHS1 carrying H1A directly fused to H2 (H1A-H2; Fig. 1C). In the first step, integration of plasmid pHS1ΩH1A-H2 by homologous recombination was selected at 42°C on agar containing gentamicin (128 μg/ml), generating a partial duplication of the locus, since the sequence of the pHS1 vector was flanked by the H1A-H2 and H1A-*erm*-H2 alleles. Serial subcultures at the permissive (28°C) and nonpermissive (42°C) temperatures in the absence of antibiotic were used to stimulate the excision and loss of pHS1ΩH1A-*erm*-H2, leaving the H1A-H2 allele in the chromosome. One clone (designated JH2-2Δ*croRS*) was obtained by screening for gentamicin and erythromycin susceptibility.

Replacement of the *erm* cassette of JH2-2Δ*croRS*/*erm* by the *croR* open reading frame was obtained by the same two-step procedure with a derivative of pHS1 carrying *croR* as a part of the H1B-H2 insert (Fig. 1D). The resulting clone, JH2-2Δ*croS*, lacked the precise *croS* open reading frame. PCR and Southern blot hybridization were used to confirm that the expected deletions from and gene replacements in JH2-2Δ*croRS*/*erm*, JH2-2Δ*croRS*, and JH2-2Δ*croS* had taken place.

Shuttle plasmids for *croS*, *croRS*, *serS*, and *pbp5* expression. The *croS* open reading frame was amplified with primers P80 and P87. Primer P80 (5′-ATCG AGGTACCGGATCCTAAAATATC*GGAGG*GTTTATT*ATG*CTCGTTAAA CCTAAAAA-3′) contained a *Kpn*I restriction site (underlined), an artificial ribosome binding site (italicized), and 20 bases complementary to the 5′ end of *croS* that included the translation initiation codon (italicized). Primer P87 (5′-ATCGATCTAGAAGATCTTT*AA*CTCTCTGATTTCTTGT-3′) contained an *Xba*I restriction site (underlined) and 20 bases complementary to the 3′ end of *croS* that included the stop codon (italicized). The *croS* open reading frame was cloned under the control of the *aphA-3p* promoter (1) in the shuttle vector pAT18 (30) to generate pRQ12(*croS*). The *serS* open reading frame was amplified with primers DS (5′-AAGAGCTCTCATTTCGTCCCAAGAATATT-3′) and ES (5′-TTTTGGTACCTTATTTAATAACTG-3′), digested with *Sac*I and *Kpn*I (underlined), and cloned under the control of the *aphA-3p* promoter to generate pYC5(*serS*). A DNA fragment containing the *rrnC-croR* intergenic region, *croR*, and *croS* (coordinates 1191 to 3315) was amplified with primer P25 (5′-AGTTCGGTACCTAAGACATGTAATAATATACCAA-3′) and P87 (described above) and cloned into pAT18 using *Bam*HI (underlined) and *Xba*I to generate pRQ13(*croRS*). Plasmid pAA15 (Arbeloa and Arthur, unpublished) contains the PBP5 open reading frame cloned downstream from *aphA-3p*. DNA sequencing was performed for all recombinant plasmids used in this study to check the accuracy of the PCRs.

Purification and analysis of mRNA. Strains of *E. faecalis* were grown in 6 ml of BHI broth to an optical density at 600 nm (OD_{600}) of 0.6. Bacteria were collected by centrifugation (12,000 × *g* for 30 s at 4°C) and treated with lysozyme and lysostaphin (GramCracker kit; Ambion Inc., Austin, Tex.), and total RNA was extracted with phenol and chloroform (RNAwiz; Ambion Inc.). RNA concentration and purity were determined by the absorbance at 260 nm (A_{260}) and the A_{260}/A_{280} ratio, respectively.

Mapping of the 5′ extremity of mRNA isolated in vivo was performed by primer extension with oligonucleotides P49 (5′-AATACTCAATAGTTCTACA ATTTC-3′), P51 (5′-CACGCCACGGGTTTGTAGCTTTGC-3′), and P76 (5′-GACTTCTTTATAGATGAATGTTT-3′). Primers (10 pmol) were end labeled with [γ-^{32}P]ATP (Perkin Elmer Life Sciences Inc., Boston, Mass.) (3,000 Ci/mmol) by using 10 U of polynucleotide kinase (primer extension system; Promega Corp., Madison, Wis.) and annealed to 30 μg of total RNA for 20 min at 58°C followed by 10 min at 20°C. Avian myeloblastosis virus reverse transcriptase (primer extension system; Promega) (1 U) was added, and incubation was continued for 30 min at 42°C. The reverse transcription products were analyzed in 6% denaturing polyacrylamide gels. DNA sequencing reactions were performed with the same primers (Sequenase version 2.0 DNA; USB Corp., Cleveland, Ohio).

Northern blot hybridization was performed with commercial denaturing and running buffers (NorthernMax; Ambion Inc.). Briefly, total RNA (30 μg) was denatured in formaldehyde loading dye for 15 min at 65°C. Electrophoresis was performed in formaldehyde denaturing gel at 5 V/cm for 3 h, and RNA was transferred by vacuum onto a nylon membrane (BrightStar-Plus; Ambion Inc.). RNA was cross-linked, using UV light at 254 nm (Stratalinker UV Cross-linker 1800; Stratagene, La Jolla, Calif.) (120,000 microjoules/cm^2), to the membrane. Prehybridization was performed overnight at 68°C (ULTRAhyb solution; Ambion Inc.). Double-stranded DNA fragments used as probes (50 ng) were labeled with [α-^{32}P]dCTP (Ready-to-Go DNA labeling beads; Amersham Pharmacia Biotech, Piscataway, N.J.) (3,000 Ci/mmol), denatured at 90°C for 10 min, and added to the prehybridization solution. Hybridization was performed overnight at 42°C. The membranes were washed (using washing solutions from a NorthernMax kit [Ambion Inc.]) twice with a low-stringency solution for 5 min at room temperature and twice with a high-stringency solution for 15 min at 90°C.

Purification of $CroR_H$. The *croR* open reading frame of *E. faecalis* JH2-2 was amplified with primers 1R61 (5′-TCATGAAAATTTTAGTTGC-3′) and 2R61His (5′-AGATCTACGAGCATCGATCTTAT-3′) containing *Bsp*HI and *Bgl*II restriction sites (underlined), respectively. The *Bsp*HI-*Bgl*II fragment was cloned into the expression vector pTRCHis60 (23) digested with *Nco*I and *Bgl*II. For protein production, *Escherichia coli* JM83 harboring plasmid pTRCHis60Ω*croR*$_H$ was grown at 37°C to an OD_{600} of 0.7 in 1 liter of BHI broth containing ampicillin (100 μg/ml). Isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 3 h at 30°C. Bacteria were harvested by centrifugation (8,000 × *g* for 20 min at 4°C), washed in 180 ml of 50 mM Tris-HCl (pH 7.5), and resuspended in 10 ml of the same buffer containing 100 mM NaCl. Bacteria were disrupted by sonication for 2 min with cooling, the extract was centrifuged at 12,000 × *g* for 30 min at 4°C, and the supernatant was mixed with 4 ml of Ni^{2+}-nitrilotriacetate-agarose resin (Amersham Pharmacia Biotech, Saclay, France) previously equilibrated with 50 mM Tris-HCl (pH 7.8). After 1 h of incubation at 4°C, the resin was recovered by centrifugation and washed with 50 mM Tris-HCl (pH 7.8) containing increasing concentrations of imidazole (20, 25, 40, 100, and 250 mM). The protein fraction eluting at 250 mM was dialyzed with 50 mM Tris-HCl (pH 7.4). Gel filtration was performed on a Superdex HR10/30 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl at a flow rate of 0.5 ml/min.

Purification of $CroS_S$. A portion of the *croS* open reading frame of *E. faecalis* JH2-2 was amplified with primers S61int4 (5′-GGTGGTCATATGCTGGTGG ATAGTACTGTCG-3′) and S61int3 (5′-GGTGGTTGCTCTTCCGCAACTCT CTGATTTCTTGTTG-3′), and the PCR product was digested with *Nde*I and *Sap*I (underlined) and cloned into pTYB1 (New England Biolabs, Frankfurt am Main, Germany) digested with the same enzymes. The resulting plasmid, pTYB1Ω*croS*$_S$, encoded a fusion protein consisting of a methionine specified by the ATG initiation codon of pTYB1, residues 145 to 393 of *croS*, and the self-cleavable C-terminal intein tag. *E. coli* ER2566 (New England Biolabs) harboring pTYB1Ω*croS*$_S$ was grown at 37°C to an OD_{650} of 0.5 in 3 liters of BHI broth containing ampicillin (300 μg/ml). IPTG was added to achieve a final concentration of 0.5 mM, and incubation was continued for 17 h at 16°C. $CroS_S$ was purified from a clarified lysate by affinity chromatography on chitin beads followed by cleavage of the fusion protein with 2-mercaptoethanol (50 mM) for 18 h at 20°C (IMPACT-CN kit; New England Biolabs). Gel filtration was performed as described above for $CroR_H$.

Protein phosphorylation assays. The kinetics of $CroS_S$ autophosphorylation was tested at 20°C in a total volume of 64 μl containing the purified protein (30 μM), [γ-^{32}P]ATP triethylammonium salt (Amersham Pharmacia Biotech) (3.4 μM; 0.37 TBq/mmol), and buffer A (50 mM Tris-HCl, 25 mM KCl, 0.5 mM $MgCl_2$, pH 7.4). Samples (12 μl) were taken at 0, 5, 10, 30, and 60 min, and the reaction was quenched by the addition of 5 μl of a solution containing 125 mM Tris-HCl (pH 6.8), 2.5% sodium dodecyl sulfate (SDS), 2 mM EDTA, 0.025% bromophenol blue, and 25% glycerol. Samples were applied directly to SDS–13.5% polyacrylamide gels. Gels were dried and subjected to autoradiography without Coomassie blue staining.

To test the transfer of the phosphate group from $CroS_S$ to $CroR_H$, phosphorylated $CroS_S$ (phospho-$CroS_S$) was prepared by incubating the protein (24 μM) with [γ-^{32}P]ATP for 60 min in a total volume of 64 μl as described above. Phospho-$CroS_S$ was separated from [γ-^{32}P]ATP by ultrafiltration (Microcon YM10; Millipore Corporation, Bedford, Mass.). $CroR_H$ (24 μM) was incubated with phospho-$CroS_S$ in buffer A (64 μl), and samples (15 μl) were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Assay for in vivo promoter activity. DNA fragments were cloned upstream from the promoterless *lacZ* reporter gene of the promoter probing vector pTCV-*lac* (24). Strains of *E. faecalis* harboring derivatives of pTCV-*lac* were grown to an OD_{600} of 0.55 in broth containing erythromycin (10 μg/ml) in addition to the drug tested for induction. Mueller-Hinton broth (Bio-Rad, Marnes-la-Coquettes, France) was used for trimethoprim, and BHI broth was used for all other drugs. Bacteria were collected by centrifugation and permeabilized with toluene. The β-galactosidase activity was expressed in arbitrary units calculated according to the equation $10^3 \times$ {(the OD_{420} value of the reaction mixture) − (1.75 × the OD_{550} value)/[the time of the reaction (in minutes) × the OD_{600} value of the quantity of cells used in the assay]}, as described previously (24).

Analysis of PBPs. The technique used for the analysis of PBPs of the different strains was employed as previously described (31) except that labeling was performed with 40 μg of benzyl[^{14}C]penicillin potassium (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England)/ml (2.11 GBq/mmol).

Peptidoglycan structure analysis. Preparation and structure assignment of muropeptides by mass spectrometry were performed as previously described (6). Briefly, bacteria were grown at 37°C in BHI broth to an optical density of 0.8. Peptidoglycan was extracted with 8% SDS at 100°C, treated with pronase (200 μg/ml) and trypsin (200 μg/ml), and digested with lysozyme (200 μg/ml) and mutanolysin (200 μg/ml). Muropeptides were reduced with sodium borohydrate and separated by reverse-phase high-performance liquid chromatography (rpHPLC) on a C_{18} column (Interchrom, Monluçon, France) (3 μm; 4.6 by 250 mm) at a flow rate of 0.5 ml/min with a 0 to 20% gradient applied at between 10 and 90 min (buffer A, 0.05% trifluoroacetic acid in water; buffer B, 0.035% trifluoroacetic acid in acetonitrile [per volume]). The relative abundance of muropeptides was estimated according to the percentage of the integrate area of peaks detected by the absorbance at 210 nm. Mass spectral data were collected with an electrospray time-of-flight mass spectrometer operating in the positive mode (Qstar Pulsar I; Applied Biosystems, Courtaboeuf, France) directly connected to the C_{18} column (flow rate, 0.5 ml/min). The data were acquired with a capillary voltage of 5,200 V and a declustering potential of 20 V. The mass scan range was from *m/z* 400 to *m/z* 2,500, and the scan cycle was 1 s.

Preparation and analysis of the cytoplasmic peptidoglycan precursors. Bacteria were grown to an OD_{650} of 0.7 and treated with vancomycin (100 μg/ml) for 15 min. Peptidoglycan precursors were extracted with formic acid (1.1 M) as previously described (4) and analyzed by rpHPLC with a μ-Bondapak C_{18} column (Waters, Milford, Mass.) (3.0 by 250 mm) at a flow rate of 0.5 ml/min with 50 mM ammonium formiate (pH 3.8). A methanol gradient (0 to 20%) was applied between 24 and 44 min, and elution with 20% methanol was continued for 10 min. The relative abundance of the UDP-MurNAc-peptide was estimated according to the percentage of the integrate area of peaks detected with the absorbance at 262 nm. For mass spectral analysis, products isolated by rpHPLC were lyophilized and dissolved in a solution containing acetonitrile (49.5%) and formic acid (0.5%). The samples were desalted by rpHPLC with isocratic elution (50% acetonitrile) at a flow rate of 10 μl/min. Tandem mass spectrometry analyzes were performed with a cone voltage of 55V and with argon as the collision gas at a pressure of 15 lb/in^2 (energy, 20 to 50 eV) as previously described (6).

RESULTS

Role of the *croRS* locus in ceftriaxone resistance. The *croRS* locus of *E. faecalis* encoded a putative response regulator (CroR) belonging to the OmpR-PhoB subclass and a putative sensor kinase (CroS) containing two clusters of hydrophobic amino acid residues that might correspond to transmembrane segments (Fig. 1A). The *croRS* locus was flanked by a copy of an rRNA gene cluster (*rrnC*) and a putative seryl-tRNA synthetase gene (*serS*). Deletions were made (using the suicide vector pHS1) from the *croRS* locus by allele exchange. In the first mutant, JH2-2Δ*croRS*/*erm* (Fig. 1B), the sequence encoding a large C-terminal portion of CroR and the entire *croS* open reading frame was replaced by an *erm* erythromycin resistance cassette. The mutant retained the first 45 codons of *croR*. Deletion of a larger portion of *croR* by homologous recombination was not attempted, since this would have required the use of a significant portion of *rrnC* which is repeated in the three other rRNA clusters. JH2-2Δ*croRS* was obtained by removing the *erm* cassette from the chromosome of JH2-2Δ*croRS*/*erm* (Fig. 1C). Replacement of the cassette by the *croR* open reading frame generated JH2-2Δ*croS*, which differed from wild-type JH2-2 by a precise deletion of the *croS* gene (Fig. 1D).

The *croRS* and *croS* deletions led to a 4,000-fold decrease in the MIC of ceftriaxone and a 4-fold decrease in the MIC of ampicillin (Table 1). Deletion of *croRS* also led to similarly large (>100-fold) decreases in the MICs of expanded-spectrum cephalosporins (e.g., cefuroxime and cefepime) and moderate (2- to 8-fold) decreases in the MICs of other β-lactams (e.g., cephalothin, imipenem, amdinocillin, and oxacillin). A similar phenotype was observed following deletion of the *pbp5* gene from the chromosome of JH2-2 (JH2-2Δ*pbp5*; Arbeloa and Arthur, unpublished). A *trans*-complementation of the *croRS* deletion was obtained with a DNA fragment containing the *rrnC-croR* intergenic region, *croR*, and *croS* cloned into the replicative vector pAT18 (Table 1). Expression of *croS* alone under the control of a heterologous promoter (*aphA-3p*) complemented the *croS* deletion. Thus, the *croR* and *croS* genes were both required for intrinsic β-lactam resistance.

TABLE 1. Susceptibility of *E. faecalis* strains to β-lactam antibiotics

Strain	Plasmid[a]	MIC (μg/ml) of:	
		Ceftriaxone	Ampicillin
JH2-2	None	1,000	2
JH2-2Δ*pbp5*	None	0.25	0.5
JH2-2Δ*croRS*	None	0.25	0.5
JH2-2Δ*croS*	None	0.25	0.5
JH2-2Δ*croRS*	pRQ13 (*croRS*)	256	2
JH2-2Δ*croS*	pRQ13 (*croRS*)	512	2
JH2-2Δ*croRS*	pRQ12 (*croS*)	0.25	0.5
JH2-2Δ*croS*	pRQ12 (*croS*)	1,000	2
JH2-2Δ*croRS*	pYC5 (*serS*)	0.25	0.5
JH2-2Δ*croRS*	pAA15 (*pbp5*)	0.5	0.5
JH2-2Δ*pbp5*	pAA15 (*pbp5*)	1,000	2

[a] The *croRS* genes were expressed under the control of the native *croRp* promoter in pRQ13. The *croS*, *serS*, and *pbp5* open reading frames were expressed under the control of the heterologous *aphA-3p* promoter in pRQ12, pYC5, and pAA15, respectively.

Deletion of *croS* and *croRS* was not associated with modification of the pattern of PBPs labeled with benzyl[^{14}C]penicillin (data not shown). Expression of *pbp5* under the control of *aphA-3p* did not restore β-lactam resistance in JH2-2Δ*croRS* (Table 1) despite overproduction of PBP5. These results show that low-affinity PBP5 was produced but could not mediate ceftriaxone resistance in JH2-2Δ*croRS*. Expression of the *serS* seryl tRNA synthetase gene under the control of *aphA-3p* did not restore ceftriaxone resistance in JH2-2Δ*croRS* (Table 1). The role of the latter gene was investigated in the present study, since two-component regulatory systems frequently regulate adjacent genes.

Phosphotransfer reactions catalyzed by purified $CroS_S$ and $CroR_H$. A soluble fragment of CroS lacking the two putative *trans*-membrane segments of the protein was produced in *E. coli* as a translational fusion containing a C-terminal intein tag. Following affinity purification on chitin beads and cleavage of the tag, the soluble fragment of the sensor kinase (designated $CroS_S$) was expected to differ from CroS by the absence of the first 144 amino acid residues and the presence of an additional methionine introduced for translation initiation. Analysis of purified $CroS_S$ (28,183 Da, 250 residues) by SDS-PAGE showed a 31-kDa protein band estimated to be 95% pure (3 mg of protein per liter of culture). Gel filtration under the conditions described in Materials and Methods revealed a protein peak with an estimated mass of 60 kDa, indicating that $CroS_S$ eluted as a dimer. No protein was detected at the elution volume expected for the monomer.

Full-length CroR fused to a C-terminal six-histidine tag (Ser-Arg-His_6) was purified by affinity chromatography on a nickel column. The protein (designated $CroR_H$) was judged using SDS-PAGE to be more than 95% pure and had an

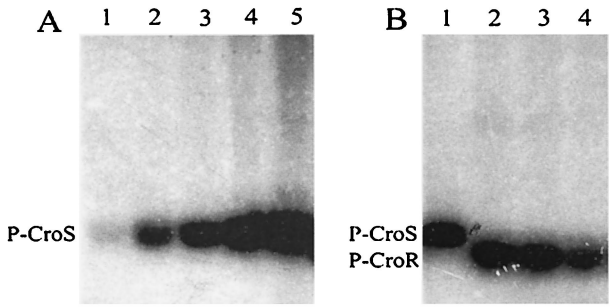


FIG. 2. Phosphotransfer reactions catalyzed by $CroS_S$ and $CroR_H$. (A) Kinetics of $CroS_S$ autophosphorylation. $CroS_S$ was incubated with [γ^{32}-P]ATP for 0, 5, 10, 30, and 60 min (lanes 1 to 5, respectively) and applied to an SDS–13.5% polyacrylamide gel. (B) Transfer of the phosphate group from the phosphorylated form of $CroS_S$ (P-CroS) to $CroR_H$. Phospho-$CroS_S$ was prepared (lane 1) and incubated with $CroR_H$ for 2, 5, and 20 min (lanes 2, 3, and 4, respectively).

estimated mass of 27 kDa (30 mg per liter of culture). $CroR_H$ eluted as a monomer in the gel filtration column.

Autophosphorylation of $CroS_S$ was assayed by incubating the purified protein with [γ^{32}-P]ATP (Fig. 2). A radioactive protein band corresponding to phospho-$CroS_S$ was detectable after 5 min of incubation and increased up to 60 min (Fig. 2A). The phospho-$CroS_S$ adduct was sufficiently stable to allow for removal of [γ-^{32}P]ATP by ultrafiltration (Fig. 2B, lane 1). Upon addition of purified $CroR_H$, the radiolabeled phosphate group was entirely transferred from $CroS_S$ to $CroR_H$ in less than 2 min. Upon further incubation, the intensity of the phospho-$CroR_H$ protein band slowly decreased due to dephosphorylation of the protein.

Mapping of mRNA isolated in vivo. Reverse transcription of mRNA isolated (using primer P49 [Fig. 3A]) from *E. faecalis* JH2-2 revealed a single putative transcriptional start site upstream from *croR* (data not shown). The product of reverse transcription was more abundant for mRNA isolated from bacteria grown in the presence of ceftriaxone at 1,000 μg/ml, and additional products were not detected. The putative transcription start site detected for both growth conditions was preceded by two hexanucleotides separated by 18 bases (TTG TCC-N_{18}-TAAAAT) resembling the −35 and −10 consensus sequences of vegetative promoters. Primer extension with oligonucleotide P76 (Fig. 3A) revealed a putative transcription start downstream from *croS* that was also preceded by sequences similar to the consensus sequence (TTGACA-N_{17}-TATTCT). The corresponding transcript extended into the *serS* open reading frame, as a ca. 370-base reverse-transcription product was observed with primer P51 (data not shown). The other two extension products (182 and 131 bases) were unlikely to correspond to transcription initiation at additional sites since they may be accounted for by mRNA processing and a reverse transcription stop at a putative transcriptional terminator, respectively, as was shown for the *serS* gene of *Bacillus subtilis* (9). In addition, the DNA fragment delineated by coordinates 3,389 to 3,643 (Fig. 3A) did not display promoter activity in the promoter probing vector pTCV-*lac* (data not shown).

Northern blot analysis of total RNA from JH2-2, JH2-2Δ*croRS*, and JH2-2Δ*croS* was performed with probes generated from internal fragments of *croR*, *croS*, and *serS* (Fig. 3B). The *croR* and *croS* probes detected the same ca. 2,400-base band in RNA preparations from JH2-2, indicating that the two genes were cotranscribed (lanes 1 and 5). Growth in the presence of ceftriaxone (1,000 μg/ml) led to the same hybridization pattern (lanes 2 and 6). As expected, the 1,161-bp *croS* deletion resulted in a decrease in the size of the RNA band detected by the *croR* probe in JH2-2Δ*croS* (lane 3). A unique 1,500-base band was detected by the *serS* probe in RNA preparations from the three strains (lanes 9 to 12), indicating that *croRS* and *serS* were transcribed independently.

In vivo activity of the *croRp* and *serSp* promoters. DNA fragments carrying the *croR* and *serS* promoters (Fig. 3A) were cloned upstream from the *lacZ* reporter gene of plasmid pTCV-*lac* and introduced into *E. faecalis* JH2-2, JH2-2Δ*croRS*, and JH2-2Δ*croS*. Control experiments were also performed with the vector alone and the heterologous *aphA-3p* promoter previously characterized in this system (24). Determination of β-galactosidase activity (Table 2) showed that the *croRp* promoter in JH2-2 was inducible by ceftriaxone. The basal level of expression of the *croRp-lacZ* transcriptional fusion appeared reduced in JH2-2Δ*croRS* (fivefold) and in JH2-2Δ*croS* (threefold) in comparison to the level seen with the JH2-2 host. The *serSp* promoter in JH2-2 did not respond to ceftriaxone, and similar β-galactosidase activity was detected in the Δ*croS* and Δ*croRS* mutants.

Representatives of various antibiotic classes were also tested for their capacity to induce the *croRp-lacZ* fusion in JH2-2 (Table 3). All β-lactam antibiotics that were tested acted as inducers, including narrow-, expanded-, and broad-spectrum cephalosporins, imipenem, ampicillin, oxacillin, and amdinocillin. Induction also occurred with inhibitors of early (phosphomycin and D-cycloserine) and late (vancomycin, moenomycin, ramoplanin, and bacitracin) steps of peptidoglycan synthesis. In contrast, no induction was observed with the aminoglycoside gentamicin (a ribosome inhibitor) and the fluoroquinolone ofloxacin (a DNA gyrase and topoisomerase IV inhibitor). The dihydrofolate reductase inhibitor trimethoprim was a weak inducer.

Ceftriaxone at low concentrations (0.05 to 0.25 μg/ml) did not induce the *croRp-lacZ* fusion in JH2-2Δ*croRS* and JH2-2Δ*croS* (data not shown). The role of CroR and CroS in induction could not be fully investigated with this drug, since the concentrations required in JH2-2 for induction were inhibitory for the mutants. For this reason, the glycosyltransferase inhibitor moenomycin was also tested, revealing induction in JH2-2 but not in JH2-2Δ*croRS* or JH2-2Δ*croS* (data not shown).

Structure of cytoplasmic peptidoglycan precursors and of muropeptides. UDP-MurNAc-peptide precursors from *E. faecalis* JH2-2 and JH2-2Δ*croRS* were compared by rpHPLC and mass spectrometry (Table 4). Deletion of the *croRS* locus was not associated with any defect in the assembly of the nucleotide UDP-MurNAc-pentapeptide, since precursors containing incomplete peptide stems were present in similar low amounts in both strains (UDP-MurNAc-tripeptide) or detected in neither strain (UDP-MurNAc-L-Ala and UDP-MurNAc-L-Ala-D-Glu). Addition of L-Ala to the ε-amino group of the pentapeptide stem of the nucleotide by the BppA1 transferase (5) resulted in similar amounts of UDP-MurNAc-hexapeptide in the Δ*croRS* mutant and in the parental strain. As expected, the

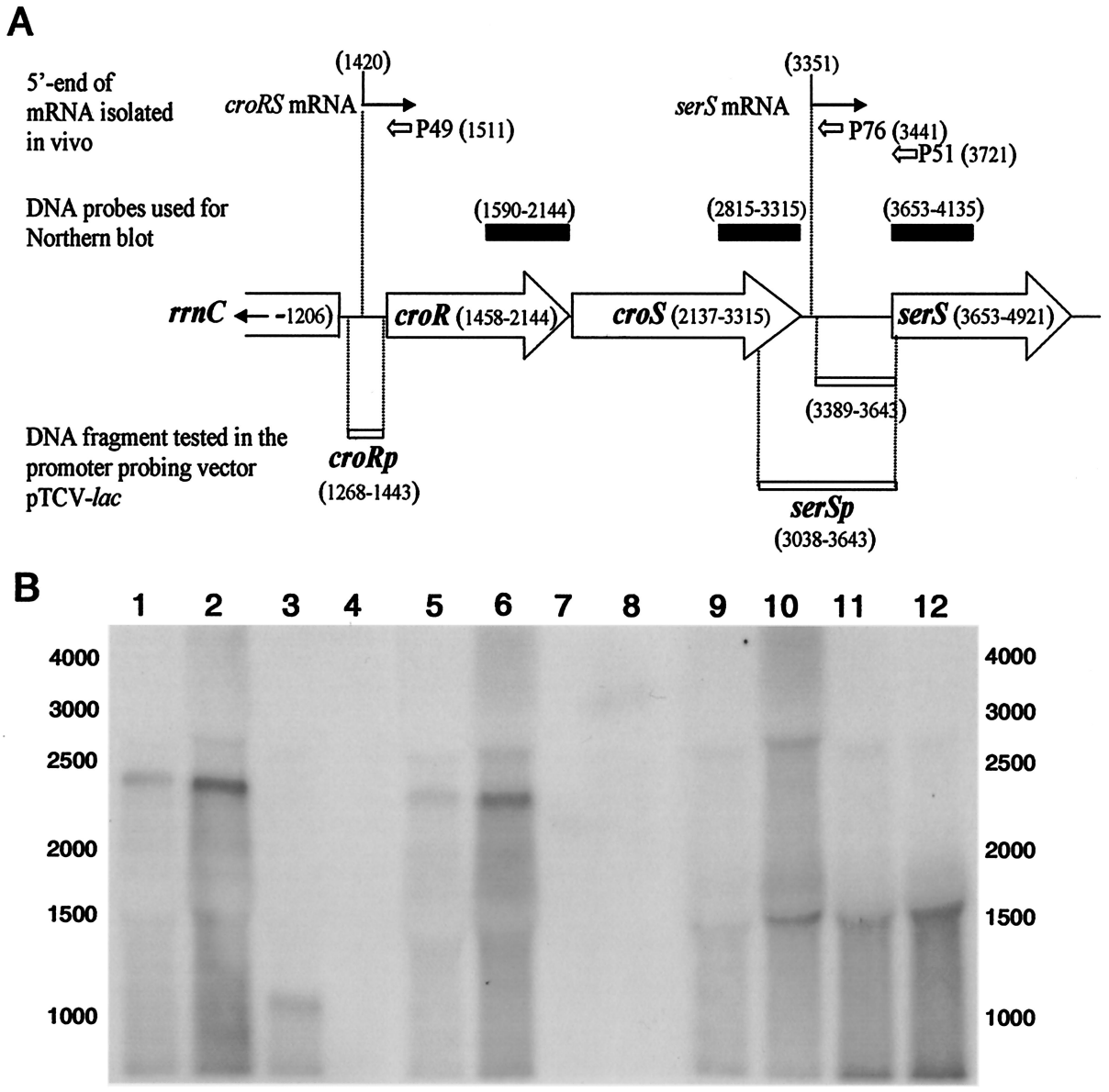


FIG. 3. Transcriptional analysis of the *croRS* locus. (A) The positions of oligonucleotides P49, P51, and P76 used for primer extension and of the DNA fragments used as probes for the Northern blot hybridizations (solid boxes) are indicated above the map of the cluster. Open boxes below the map represent the DNA fragments tested for promoter activity in pTCV-*lac*. (B) Northern blot analysis of total RNA with probes generated from internal fragments of *croR* (lanes 1 to 4), *croS* (lanes 5 to 8), and *serS* (lanes 9 to 12). Total RNA was extracted from *E. faecalis* JH2-2 grown in the absence (lanes 1, 5, and 9) or presence (lanes 2, 6, and 10) of ceftriaxone (1,000 μg/ml). RNA from JH2-2Δ*croS* (lanes 3, 7, and 11) and JH2-2Δ*croRS* (lanes 4, 8, and 12) was extracted from bacteria grown in the absence of ceftriaxone.

pools of UDP-MurNAc-hexapeptide were small, since the BppA1 transferase preferentially uses lipid intermediates as substrates (5).

The muropeptide compositions of the peptidoglycans of *E. faecalis* JH2-2 and JH2-2Δ*croRS* were also similar (Fig. 4 and data not shown). The predominant muropeptides contained two D-alanyl residues at the free C-terminal end and two L-alanyl residues both in the cross-bridge and at the free N-terminal end. Muropeptides of lesser abundance differed from the above structures by combinations of MurNAc *O*-acetylation and loss of the C-terminal D-Ala residues, as previously described (6). These results indicate that deletion of the *croRS* locus did not affect the extent or mode of peptidoglycan cross-linking in *E. faecalis* JH2-2.

DISCUSSION

The deletions from the chromosomal *croRS* locus of *E. faecalis* JH2-2 and complementation analysis indicated that the CroR response regulator and the CroS sensor kinase were required for intrinsic β-lactam resistance (Table 1). A soluble fragment of CroS and full-length CroR were purified to con-

TABLE 2. Promoter activities in various derivatives of *E. faecalis* JH2-2

Promoter fused to *lacZ*	β-Galactosidase activities (mean arbitrary units ± SD) for strain[a]:			
	JH2-2		JH2-2Δ*croRS*	JH2-2Δ*croS*
	Not induced	Induced[b]	Not induced	Not induced
croRp	5.8 ± 1.5	35.0 ± 8.0	1.3 ± 0.6	2.1 ± 0.7
serSp	6.4 ± 1.9	7.5 ± 1.1	13.5 ± 1.4	4.3 ± 0.6
aphA-3p	56.6 ± 14.0	66.4 ± 17.3	71.5 ± 4.0	76.7 ± 2.4
None	<1	<1	<1	<1

[a] Experiments were performed in triplicate, and the mean values ± standard deviation (SD) of arbitrary units of β-galactosidase-specific activities are indicated.
[b] Induction was performed with ceftriaxone at 1,000 μg/ml.

TABLE 4. Peptidoglycan precursors from *E. faecalis* JH2-2 and JH2-2Δ*croRS*

Precursor[a]	RT[b]	Mass	Relative abundance (%)	
			JH2-2	Δ*croRS*
UDP-MurNAc-tripeptide	40.0	1,007.2	0.3	0.2
UDP-MurNAc-pentapeptide	44.6	1,149.3	95.7	95.6
UDP-MurNAc-hexapeptide	49.2	1,220.3	4.0	4.2

[a] UDP-MurNAc-tripeptide, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-L-Lys; UDP-MurNAc-pentapeptide, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala; UDP-MurNAc-hexapeptide, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-L-Lys(N^{ε}-L-Ala)-D-Ala-D-Ala.
[b] RT, retention time in minutes.

firm that the proteins were functional with respect to the phosphotransfer reactions commonly catalyzed by mated kinases and response regulators of two-component regulatory systems (Fig. 2). The kinase activity of CroS may be responsible for activation of the response regulator in vivo, as found for several response regulators of the OmpR-PhoB subclass (12), although experimental evidence that phospho-CroR is the active form of the protein was not obtained in the present study. This would imply that CroR cannot be activated by cross talk, since the *croS* null mutant was susceptible to ceftriaxone.

The *croR* and *croS* genes were cotranscribed from a promoter, *croRp*, which was inducible by inhibitors of peptidoglycan synthesis in *E. faecalis* JH2-2 (Table 2). The reporter gene was expressed at a constitutive low level in JH2-2Δ*croRS* and JH2-2Δ*croS*. Thus, the adaptive response elicited by CroR and CroS involved increased transcription of the regulatory genes. This autoregulation mechanism is common to other two-component regulatory systems (16). The sensor kinases encoded by glycopeptide resistance gene clusters appear to specifically respond to vancomycin (VanB-type resistance) or to inhibitors of the transglycosylation reaction (VanA-type resistance) (2). In contrast, the CroRS system did not respond to inhibition of a specific step of peptidoglycan synthesis, since the *croRp-lacZ* transcriptional fusion was inducible by all peptidoglycan synthesis inhibitors that were tested, including compounds acting on early cytoplasmic steps, the metabolism of the lipid intermediates, transglycosylation, and transpeptidation (Table 3). A recent study of the role of two-component regulatory systems of *E. faecalis* in the response to environmental stresses suggests that the CroS sensor kinase responds to an even broader spectrum of signals (19).

TABLE 3. Induction of the *croRp-lacZ* fusion in *E. faecalis* JH2-2 by various antibiotics[a]

Antibiotic	Tested range (μg/ml)	Drug concn (μg/ml) for maximum induction	Induction ratio
Cephalothin	1–100	20	8.6
Cefuroxime	50–1,000	1,000	7.3
Ceftriaxone	20–1,000	1,000	6.0
Cefepime	100–800	200	7.8
Ampicillin	0.01–2	0.5	6.4
Amdinocillin	50–1,000	500	17.2
Imipenem	0.01–0.5	0.2	6.2
Oxacillin	1–300	100	8.0
Fosfomycin	0.01–40	40	12.9
D-Cycloserine	50–200	200	26.6
Moenomycin	0.01–0.4	0.3	8.9
Bacitracin	1–64	32	12.5
Ramoplanin	0.01–20	20	10.5
Vancomycin	0.01–5	2	4.7
Gentamicin	4–32	4	1.2
Ofloxacin	0.2–8	4	1.2
Trimethoprim	0.001–10	5	3.9

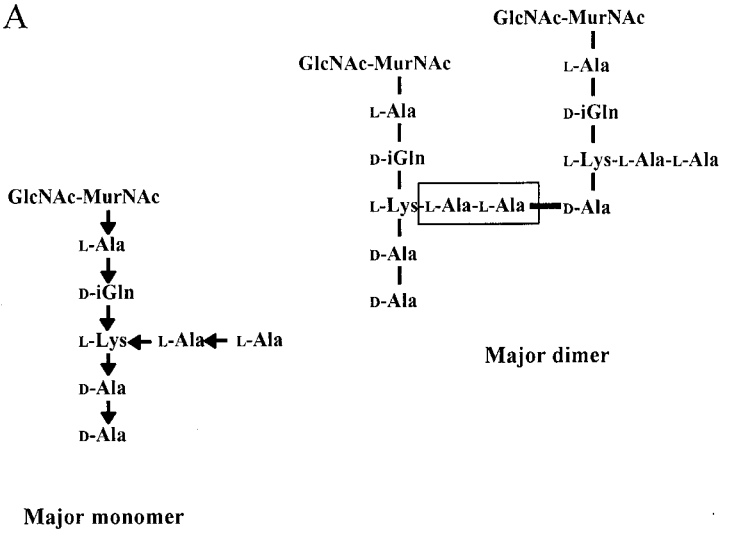
[a] Bacteria were grown to an OD_{600} of 0.55 in broth containing erythromycin (10 μg/ml) in addition to the drug tested for induction. A minimum of three concentrations were tested, and the induction ratio was calculated for the concentration leading to the highest activity.

Sequences flanking the *croRS* locus were independently transcribed, since the *rrnC* rRNA operon was in a divergent orientation and Northern blot hybridization revealed a distinct mRNA for the downstream *serS* seryl-tRNA synthetase gene. The *serSp* promoter was not inducible by peptidoglycan synthesis inhibitors and was similarly active in JH2-2, JH2-2Δ*croRS*, and JH2-2Δ*croS* (Table 2). Expression of *serS* under the control of the heterologous *aphA-3p* promoter did not restore ceftriaxone resistance in the JH2-2Δ*croRS* mutant. Thus, there is apparently no functional link between the *croRS* locus and the flanking *serS* locus. Of note, this region of the chromosome does not seem to undergo frequent recombination events, since the relative positions of *croRS* and *serS* are conserved in *E. faecalis* and in *E. faecium* (data not shown).

Deletion of *croRS* and *pbp5* had the same impact on the MICs of β-lactam antibiotics (Table 1). The patterns of PBPs labeled with benzyl[^{14}C]penicillin were similar for JH2-2, JH2-2Δ*croRS*, and JH2-2Δ*croS* (data not shown). Introduction of plasmid pAA15, harboring *pbp5* under the control of *aphA-3p*, led to overproduction of PBP5 in JH2-2Δ*croRS*, but the MIC of ceftriaxone was only marginally increased (Table 1). These results indicate that susceptibility to ceftriaxone caused by the *croRS* deletion cannot be attributed to the lack of PBP5 production.

Gram-positive cocci produce branched peptidoglycan precursors containing a side chain consisting of two L-alanyl residues in *E. faecalis* (Fig. 4A), five glycyl residues in *S. aureus*, and the sequence L-Ser-L-Ala or L-Ala-L-Ala in *S. pneumoniae* (28). The genes encoding the transferases for synthesis of the pentaglycine side chain in *S. aureus* were initially identified as factors essential for methicillin resistance (*fem*) after random mutagenesis of the *S. aureus* chromosome (3, 25). More recently, production of incomplete side chains was also reported to lead to impaired expression of acquired resistance to β-lac-

A



B

muropeptide	(mass)	Relative abundance (%) JH2-2	Δ*croRS*
monomer			
major	(1109.6)	20.9	14.7
total		26.5	23.9
dimer			
major	(2130.1)	26.4	22.0
total		40.8	40.1
trimer			
major	(3150.5)	15.1	15.2
total		22.4	28.3
tetramer			
major	(4171.1)	7.0	4.3
total		10.3	7.8

FIG. 4. Muropeptides from *E. faecalis* JH2-2 and JH2-2Δ*croRS.* (A) The most abundant muropeptides of both strains contained two D-Ala residues at the free C-terminal end and two L-Ala residues both at the free N-terminal end and in the cross-bridge (boxed). D-Glutamic acid is incorporated into the precursors and secondarily α-amidated (D-iGln, γ-D-glutaminyl residue). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid. The orientation of the CO→NH peptide bonds is indicated by arrows. (B) Relative abundances of the major monomer, dimer, trimer, and tetramer. The totals include muropeptides of lesser abundance lacking the two C-terminal D-Ala residues and *O*-acetylated derivatives.

tam antibiotics in *S. pneumoniae* (13) and of intrinsic resistance to ceftriaxone in *E. faecalis* (6). The screening for impaired expression of methicillin resistance in *S. aureus* also identified mutations in genes encoding enzymes for the assembly of the nucleotide precursor UDP-MurNAc-pentapeptide (17, 21). In the latter case, the mutations could affect the amount of precursor produced rather than its structure (10). Since (despite the production of PBP5) deletion of the *croRS* locus was associated with ceftriaxone susceptibility, the structures of cytoplasmic precursors and muropeptides were analyzed to screen for defects in the production of the substrate of PBP5. Deletion of *croRS* was not associated with accumulation of nucleotide precursors containing incomplete stem peptides (Table 4). Further, synthesis of the L-alanyl-L-alanine side chain was not impaired since the mutant produced wild-type levels of UDP-MurNAc-hexapeptide (Table 4) and two L-alanyl residues were present both in the cross-bridge and in the free N-terminal end of the muropeptides (Fig. 4). Finally, the relative proportions of monomer, dimer, trimer, and tetramer were similar in JH2-2Δ*croRS* and in the parental strain. These results indicate that PBP5 did not mediate ceftriaxone resistance in JH2-2Δ*croRS*, despite delivery of an apparently unaltered supply of disaccharide-peptide subunits to the peptidoglycan polymerization complexes.

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