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 rrmC-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 ppp5 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-Ala4-L-Ala-Lys3 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.

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,
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'-ATTGGATTTCTGATATCGC C-3' and 5'-AGATCTCCTTGGTTTGTTGGCC-3'; for H1B, primers 5'-ATT GATTTCCTGATATCGC-3' and 5'-TTTGGATATCTTTAAACGACGATCGCTT TAT-3'; and for H2, primers 5'-AGATCTGAGTTAATTGACACCC-3' and 5'-GCAGACACATCATCCG-3') containing BglII restriction sites (underlined) to facilitate subsequent cloning steps. The fragments were cloned (with or without an intervening BglII 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 an erythromycin resistance gene cassette (erm), JH2-2ΔcroRS/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. To construct JH2-2ΔcroRS, the erm cassette was deleted from the chromosome of JH2-2ΔcroRS/erm by allele exchange with H1A directly linked to H2. (D) To construct JH2-2ΔcroS, the erm cassette of JH2-2ΔcroRS/erm was replaced (using H1B linked to H2) by the croR open reading frame. Numbers in parentheses indicate the extents of the deletions from JH2-2ΔcroRS and from JH2-2ΔcroS. Coordinate 1 corresponds to position 3,169,253 of the assembled E. faecalis genome at www.tigr.org.

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 rnc rRNA gene cluster (open box). BglII restriction sites were introduced at one extremity of H1A, H1B, and H2. (B) Replacement of croRS by an erythromycin resistance gene cassette (erm). JH2-2ΔcroRS/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 JH2-2ΔcroRS, the erm cassette was deleted from JH2-2ΔcroRS/erm by allele exchange with H1A directly linked to H2. (D) To construct JH2-2ΔcroS, the erm cassette of JH2-2ΔcroRS/erm was replaced (using H1B linked to H2) by the croR open reading frame. Numbers in parentheses indicate the extents of the deletions from JH2-2ΔcroRS and JH2-2ΔcroS. Coordinate 1 corresponds to position 3,169,253 of the assembled E. faecalis genome at www.tigr.org.
using a derivative of pH1 carrying H1A directly fused to H2 (H1A-H2; Fig. 1C). In the first step, integration of plasmid pH1S11H1A-H2 by homologous recombination was selected on 42°C on agar containing gentamicin (125 μg/ml), generating a partial duplication of the locus, since the sequence of the pH1 vector was flanked by H1A-H2 and H1A-erm-H2 alleles. Sequences at the permisive (28°C) and nonpermissive (42°C) temperatures in the absence of antibiotic were used to stimulate the excision and loss of pH1S11H1A-erm-H2, leaving the H1A-H2 allele in the chromosome. One clone (designated JH2-2Δcro2rs) was obtained by screening for gentamicin and erythromycin sensitivity.

Replacement of the erm cassette of JH2-2Δcro2rs by the cro2 open reading frame was obtained by the same two-step procedure with a derivative of pH1 carrying cro2 as a part of the H1B-H2 insert (Fig. 1D). The resulting clone, JH2-2Δcro2rs, lacked the precise cro open reading frame. PCR and Southern blot hybridization were used to confirm the expected deletions from gene replacement in JH2-2Δcro2rs, JH2-2Δcro2rs, and JH2-2Δcro2rs had taken place.

Shuttle plasmids for croS, croRS, serS, and php5 expression. The croS open reading frame was amplified with primers P80 and P87. Primer P80 (5′-ATCGATTTTAGGTTTATTTGCTCTGTTAATAGTTCTACA-3′) contained an XbaI restriction site (underlined) and 20 bases complementary to the 3′ end of croS that included the translation initiation codon (italicized). Primer P87 (5′-GATCACGCGAGCTTACTTATTTAATAACTG-3′) had 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-3 promoter (1) in the shuttle vector pAT18 (30) to generate pQ12(croS). The serS open reading frame was amplified with primers DS (5′-AGAAGCTTCTATTGCGGAAATATT-3′) and Es (5′-TTTTGGTACCTTATTTAATAACTG-3′), digested with SphI (underlined), and cloned under the control of the aphA-3 promoter to generate pYC5(serS). A DNA fragment containing the rmc-croS intergenic region, croS, and croS (coordinates 1191 to 3315) was amplified with primer P25 (5′-AGTCTGGATCAAGCTTACTAATATACCA-3′) and P87 (described above) and cloned into pAT18 using BamHI (underlined) and XbaI to generate pQ13(croS). Plasmid pAA15 (Arbeloa and Arthur, unpublished) contains the PBP5 open reading frame cloned downstream from the ATG initiation codon of plasmid pH1S11 carrying pHP5 expression. croS was amplified and cloned into pTYB1 (New England Biolabs, Frankfurt am Main, Germany) digested with the same enzymes. The resulting plasmid, pTYB1croS, encoded a fusion protein consisting of a methionine-specific ATG initiation codon of pTYB1 residues 143 to 393 of croS, and the self-cleavable C-terminal intein tag. E. coli ER2566 (New England Biolabs) harboring pTYB1croS was grown at 37°C to an OD600 of 0.5 in 3 liters of BHI broth containing ampicillin (100 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 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 1 h at 20°C (IMPACT-CN kit; New England Biolabs). Gel filtration was performed as described above for croRf1.

Protein phosphorylation assays. The kinetics of croS autophosphorylation was tested at 20°C in a total volume of 64 μl containing the purified protein (30 μM), [γ-32P]ATP triethylammonium salt (Amersham Pharmacia Biotech) (3 μCi/μl, 0.37 TBq/μmol), and buffer A (50 mM Tris-HCl, 5 mM KCl, 0.5 mM MgCl2, 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–15% polyacrylamide gels. Gels were dried and subjected to autoradiography without Coomassie blue staining.

To test the transfer of the phosphate group from croS to croRf1, phosphorylated croS (phospho-croS) was prepared by incubating the protein (24 μM) with [γ-32P]ATP for 60 min in a total volume of 64 μl as described above. Phospho-croS was separated from [γ-32P]ATP by ultrafiltration (Micronen YM10; Millipore Corporation, Bedford, Mass.). croRf1 (24 μM) was incubated with phospho-croS, 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 pTVC-lac (24). Strains of E. faecalis harboring derivatives of pTVC-lac were grown to an OD600 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-Coquette, France) was used for E. coli, 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 × [(the OD420 value of the reaction mixture) – (1.75 × the OD420 value) × the time of the reaction (in minutes) × the OD420 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 of E. faecalis was employed as described (31) except that the gel was performed with 40 μg of benzylpenicillin (ammonium) (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 BH1 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 lysoylm (200 μg/ml) and mutanolysin (200 μg/ml). Muropeptides were reduced with sodium borohydride and separated by reverse-phase high-performance liquid chromatography (rpHPLC) on a C18 column (Interchrom, Montluçon, France) (3 μm; 4.6 by 250 mm) at a flow rate of 0.5 ml/min with a 0 to 20% gradient applied between 10 and 90 min (buffer A, 0.05% trifluoroacetic acid in water; buffer B, 0.05% 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 C18 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 OD600 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 C18 column (Waters, Milford, Mass.) (3.0 by 250 mm) at a flow rate of 0.5 ml/min with 50 mM ammonium formate (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 according to the percentage of the integrate area of peaks detected with 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 C18 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.

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, JH2-2ΔcroRS/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. JH2-2ΔcroS/erm was obtained by removing the erm cassette from the chromosome of JH2-2ΔcroRS/erm (Fig. 1C). Replacement of the cassette by the croR open reading frame generated JH2-2ΔcroS, which differed from wild-type JH2-2 by the 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 pphS gene from the chromosome of JH2-2 (JH2-2ΔpphS; 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.

Deletion of croS and croRS was not associated with modification of the pattern of PBP s labeled with benzyl[14C]penicillin (data not shown). Expression of pphS under the control of aphA-3p did not restore β-lactam resistance in JH2-2ΔcroRS (Table 1) despite overproduction of PBP5. These results show that low-affinity PBP5 was produced but could not mediate ceftriaxone resistance in JH2-2ΔcroRS. Expression of the seryl tRNA synthetase gene under the control of aphA-3p did not restore ceftriaxone resistance in JH2-2ΔcroRS (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 and CroRq. 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 CroS4) 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 CroS4 (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 CroS4 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-His6) was purified by affinity chromatography on a nickel column. The protein (designated CroRq) was judged using SDS-PAGE to be more than 95% pure and had an

<table>
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<th>Strain</th>
<th>Plasmid</th>
<th>MIC (μg/ml) of:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td>JH2-2</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>JH2-2ΔpphS</td>
<td>pAA15 (pphS)</td>
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*The croS genes were expressed under the control of the native croR promoter in pRQ13. The croS, serS, and pphS open reading frames were expressed under the control of the heterologous aphA-3p promoter in pRQ12, pYC5, and pAA15, respectively.*
bacteria grown in the presence of ceftriaxone at 1,000
transcription was more abundant for mRNA isolated from
-32P\]ATP by ultrafiltration (Fig. 2B, lane 1).

Autophosphorylation of CroS\(_5\) was assayed by incubating
the purified protein with [\(\gamma^32\)-P\]ATP (Fig. 2). A radioactive
protein band corresponding to phospho-CroS\(_5\) was detectable
after 5 min of incubation and increased up to 60 min (Fig. 2A).
The phospho-CroS\(_5\) adduct was sufficiently stable to allow for
removal of [\(\gamma^32\]-P\]ATP by ultrafiltration (Fig. 2B, lane 1).
Upon addition of purified CroR\(_{H}\), the radiolabeled phosphate
group was entirely transferred from CroS\(_5\) to CroR\(_{H}\) in less
than 2 min. Upon further incubation, the intensity of the phospho-
CroR\(_{H}\) protein band slowly decreased due to dephosphory-
lation 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 up-
stream 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 \(\mu\)g/ml,
and additional products were not detected. The putative tran-
scription start site detected for both growth conditions was
preceded by two hexanucleotides separated by 18 bases (TTG
CAAAT) 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 termi-
nator, respectively, as was shown for the serS gene of Bacillus
subtilis (9). In addition, the DNA fragment delineated by co-
ordinates 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-
2ΔcroR\(_S\), and JH2-2ΔcroS\(_S\) was performed with probes gener-
ated 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 pre-

cence of ceftriaxone (1,000 \(\mu\)g/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 JH2-2ΔcroS (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, JH2-2ΔcroR\(_S\),
and JH2-2ΔcroS\(_S\). 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 pro-
moter in JH2-2 was inducible by ceftriaxone. The basal level of
expression of the croRp-lacZ transcriptional fusion appeared
reduced in JH2-2ΔcroS (fivefold) and in JH2-2ΔcroS\(_S\) (three-
fold) 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 ΔcroS
and ΔcroRS 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 amdinocilin.
Induction also occurred with inhibitors of early (phos-
phomycin and d-cycloserine) and late (vancomycin, moenomyc-
in, ramoplanin, and bacitracin) steps of peptidoglycan
synthesis. In contrast, no induction was observed with the ami-
noglycoside gentamicin (a ribosome inhibitor) and the fluoro-
quinolone 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 \(\mu\)g/ml) did not
induce the croRp-lacZ fusion in JH2-2ΔcroRS and JH2-2
ΔcroS\(_S\) (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 inhibi-
tor moenomycin was also tested, revealing induction in JH2-2
but not in JH2-2ΔcroRS or JH2-2ΔcroS\(_S\) (data not shown).

Structure of cytoplasmic peptidoglycan precursors and of
muropeptides. UDP-MurNac-peptide precursors from "E. faecalis"
JH2-2 and JH2-2ΔcroRS 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 in-
complete 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 pentapep-
tide stem of the nucleotide by the BppA1 transferase (5) re-
sulted in similar amounts of UDP-MurNac-hexapeptide in the
ΔcroRS mutant and in the parental strain. As expected, the
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 JH2-2/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-
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 JH2-2 ΔcroRS and JH2-2 ΔcroS. 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).

Sequences flanking the croRS locus were independently transcribed, since the rrmC 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, JH2-2 ΔcroRS, and JH2-2 ΔcroS (Table 2). Expression of serS under the control of the heterologous aphA-3p promoter did not restore ceftriaxone resistance in the JH2-2 ΔcroRS 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[14C]penicillin were similar for JH2-2, JH2-2 ΔcroRS, and JH2-2 ΔcroS (data not shown). Introduction of plasmid pAA15, harboring pbp5 under the control of aphA-3p, led to overproduction of PBPs in JH2-2 ΔcroRS, 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 PBPs 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 1-Ser-1-Ala or 1-Ala-1-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-lact-
tam antibiotics in *S. pneumoniae* (13) and of intrinsic resistance to ceftriaxone in *E. faecalis* (6). The screening for impaired expression of methylillin 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 JH2-2ΔcroRS and in the parental strain. These results indicate that PBP5 did not mediate ceftriaxone resistance in JH2-2ΔcroRS, despite delivery of an apparently unaltered supply of disaccharide-peptide subunits to the peptidoglycan polymerization complexes.

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