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Resistance to Bacitracin as Modulated by an *Escherichia coli* Homologue of the Bacitracin ABC Transporter BcrC Subunit from *Bacillus licheniformis*

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A small open reading frame from the *Escherichia coli* chromosome, $bcrC_{EC}$, encodes a homologue to the BcrC subunit of the bacitracin permease from *Bacillus licheniformis*. We show that disruption of the chromosomal $bcrC_{EC}$ gene causes bacitracin sensitivity and, conversely, that $BcrC_{EC}$ confers bacitracin resistance when expressed from a multicopy plasmid.

Bacitracin is an antibiotic produced by certain species of *Bacillus* as a mixture of related cyclic polypeptides. Its primary mode of action is interference with bacterial cell wall synthesis through inhibition of dephosphorylation of the peptidoglycan carrier C_{55}-isoprenyl pyrophosphate (IPP) (14). In *Bacillus licheniformis*, an active ABC-type efflux system comprised of three proteins, BcrA, BcrB, and BcrC, has been shown to be responsible for the resistance of the cells to bacitracin (10). Being a potent antimicrobial drug, bacitracin is used clinically in combination with other antibiotics. However, despite its clinical importance, little is known about the mechanisms by which bacteria other than *B. licheniformis* acquire resistance to this antibiotic. Only indirect mechanisms of bacitracin resistance, involving IPP, have been described for *Escherichia coli* and other gram-negative bacteria (4, 11).

Recently, we have isolated an *E. coli* genomic clone (P2) encoding a multidrug resistance protein (MdfA) with a broad spectrum of drug specificity (6) (also termed Cmr [9]). The DNA sequence of *E. coli* between *mdfA* and *deoR* near min 19 revealed an open reading frame of 198 codons (*orf1*, termed here $bcrC_{EC}$). In agreement with the work of Blattner et al. (3), $bcrC_{EC}$ encodes a putative hydrophobic protein (termed here $BcrC_{EC}$) that exhibits approximately 30% identity to one of the integral membrane subunits of the *B. licheniformis* bacitracin resistance complex (BcrC) (10). Accordingly, the hydrophobicity profiles of BcrC from *B. licheniformis* and its homologue from *E. coli* are nearly identical along most of their primary sequences (data not shown). This suggests a common function. In addition, no significant sequence matches were found with other known proteins, whereas $BcrC_{EC}$ showed approximately 80% identity to a putative permease from *Salmonella typhimurium*, described as a lipoprotein similar to bacitracin permease from *B. licheniformis* (15). Here we report that $BcrC_{EC}$ modulates bacitracin resistance in *E. coli*.

In order to characterize the product of the $bcrC_{EC}$ gene, plasmid pT-$bcrC_{EC}$ was constructed by deleting sequences from the P2 plasmid, leaving intact $bcrC_{EC}$ and its own promoter and also an appropriately oriented T7 promoter (Fig. 1). We then proceeded to verify that the $bcrC_{EC}$ gene product is a transmembrane protein, as expected from its structure. To get a detectable amount of protein, the plasmid pT-$bcrC_{EC}$ was first transformed into *E. coli* BL21(DE3), and the gene was expressed from the T7 promoter in the presence of [^{35}S]methionine. Membrane fractions were then prepared as described elsewhere (17), and proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A radiolabeled protein with the expected molecular mass for $BcrC_{EC}$ (23 kDa) was observed in the sample from membrane fractions of isopropyl-β-D-thiogalactopyranoside-induced cells, thus confirming the anticipated membrane localization (data not shown).

The putative involvement of $BcrC_{EC}$ in bacitracin resistance was studied by chromosomal $bcrC_{EC}$ gene disruption and by mild overexpression of $BcrC_{EC}$ from pT-$bcrC_{EC}$. Disruption of the chromosomal $bcrC_{EC}$ was achieved by insertion of a kanamycin resistance cassette from plasmid pUC-4K into the unique *Bsa*BI site present in $bcrC_{EC}$ (Fig. 1). The new plasmid pT-$bcrC_{EC}$::*kan* was linearized, and the $bcrC_{EC}$::*kan* allele was transferred to the chromosome of JC7623 (*recBC sbcB*) by homologous recombination. The replacement of $bcrC_{EC}$ by $bcrC_{EC}$::*kan* in Kan^r Amp^s transformants was verified by PCR analysis. The $bcrC_{EC}$::*kan* mutation was transferred to *E. coli* UT5600 by P1 transduction (1, 16). In order to test the effect of mild overexpression of $BcrC_{EC}$, wild-type and mutant cells were transformed by pT-$bcrC_{EC}$ or pT-$bcrC_{EC}$::*kan*. Resistance to bacitracin was tested by plating diluted samples of overnight cultures on solid Luria-Bertani medium containing various concentrations of bacitracin (Fig. 2).

The results show that cells harboring the chromosomal $bcrC_{EC}$ disrupted gene were two times more sensitive to bacitracin than were wild-type cells (Fig. 2a; MICs of 60 and 120 U/ml, respectively). Furthermore, it was possible to complement this increased sensitivity by transformation with pT-$bcrC_{EC}$ but not by transformation with pT-$bcrC_{EC}$::*kan*. This fact indicates that the bacitracin resistance phenotype is specifically related to the status of the $bcrC_{EC}$ gene. The same magnitude of bacitracin sensitization was observed when the $bcrC_{EC}$::*kan* allele was transferred into a *galU* derivative of UT5600, UTL2 (2, 5), 100 times more sensitive to bacitracin because of an altered cell envelope (data not shown). This result suggests that the resistance mediated by $BcrC_{EC}$ is likely to be independent of an intact outer membrane. We note that resistance to other drugs such as vancomycin, chloramphenicol, and tetraphenylphosphorium was not affected in all cases (data not shown).

Overexpression of $BcrC_{EC}$ from its own promoter on a multicopy plasmid allowed the cells to resist up to 200 U of baci-

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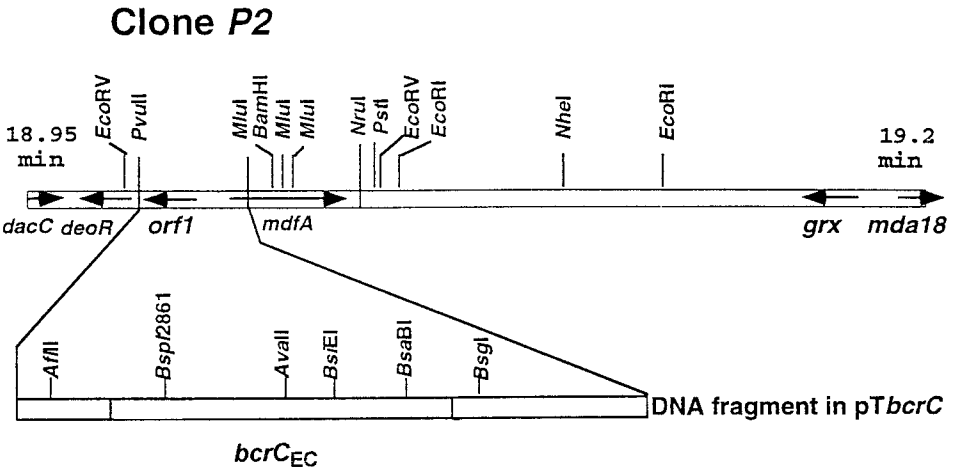

FIG. 1. Gene arrangements in the plasmids P2 and pT-*bcrC*$_{EC}$. (Upper panel) Organization of the chromosomal *E. coli* DNA clone P2. (Lower panel) DNA fragment in plasmid pT-*bcrC*$_{EC}$.

tracin per ml (Fig. 2a; drug concentration enabling 1% survival), independently of the carried chromosomal allele, either *bcrC*$_{EC}^{+}$ or *bcrC*$_{EC}$::*kan*, thus increasing the resistance to bacitracin by a factor of two or three, respectively. Similar effects of increased resistance were observed when BcrC$_{EC}$ was overexpressed in the *bcrC*$_{EC}$::*kan galU* derivative (data not shown).

The modest magnitude of the effect of overexpression of BcrC$_{EC}$ may be explained in various ways. For example, since it shows identity with a subunit of the bacitracin pump from *B. licheniformis*, we may expect the transmembrane BcrC$_{EC}$ protein to be part of a bacitracin ABC transporter and therefore to interact with BcrA and BcrB analogs. The low concentration of these proteins, chromosomally encoded but not overexpressed in our assay, would be responsible for the low effect. However, an extensive search for such molecules by sequence comparisons on the complete chromosome has revealed no obvious BcrB homologues in *E. coli*. Alternatively, BcrC$_{EC}$ could interact heterologously with another ABC system, contributing to substrate recognition and hijacking the energy-utilizing subunit for active export (12). Another possibility, although unlikely, is that BcrC$_{EC}$ acts alone as a monomer or homooligomer, with a low rate of transport. A prediction of the pump model is that the modest increase in the bacitracin extrusion rate would be unnoticeable in the *galU* permeable mutant, being masked by the high entry rate of bacitracin. However, the UTL2 mutant showed the same response as the UT5600 wild type, which makes this transport model less likely. Another model would postulate a partial protection of the bacitracin target, IPP, by BcrC$_{EC}$ protein, altering the structure of the pyrophosphate end or facilitating the access of pyrophosphatase to its substrate in the presence of bacitracin. Lastly, we cannot exclude the possibility that the observed effects are only secondary consequences of BcrC$_{EC}$ protein action on the cell metabolism.

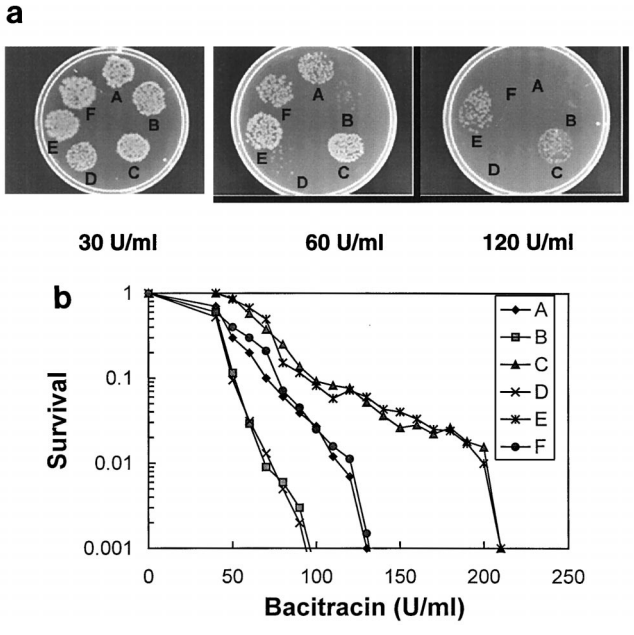

FIG. 2. Bacitracin resistance of UT5600 and its derivatives. Samples (5 μl) of a 10^4 dilution of overnight cultures of *E. coli* UT5600 and derivatives were spotted onto solid Luria-Bertani medium containing indicated concentrations of bacitracin and incubated at 37°C overnight. (a) Photographs. (b) Relative numbers of developed colonies on the various spots (averages of five experiments). A, *bcrC*$_{EC}^{+}$; B, *bcrC*$_{EC}$::*kan*; C, *bcrC*$_{EC}$::*kan*/pT-*bcrC*$_{EC}$; D, *bcrC*$_{EC}$::*kan*/pT-*bcrC*$_{EC}$::*kan*; E, *bcrC*$_{EC}^{+}$/pT-*bcrC*$_{EC}$; F, *bcrC*$_{EC}^{+}$/pT-*bcrC*$_{EC}$::*kan*.

In summary, the identity between BcrC$_{EC}$ and a subunit from the *B. licheniformis* bacitracin pump suggested to us a possible involvement of this protein in *E. coli* resistance to bacitracin, as indeed was verified in our study. The nature of this modulation of antibiotic resistance remains, however, unknown.

Nucleotide sequence accession number. The *bcrC*$_{EC}$ GenBank-EMBL-DDBJ accession no. is U00096, f198.

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