JOURNAL OF BACTERIOLOGY, Nov. 2007, p. 8361–8365 Vol. 189, No. 22
0021-9193/07/$08.00+0 doi:10.1128/JB.01028-07

The *Escherichia coli* Yej Transporter Is Required for the Uptake of Translation Inhibitor Microcin C[▽]

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Received 29 June 2007/Accepted 28 August 2007

Microcin C (McC), a peptide-nucleotide antibiotic, targets aspartyl-tRNA synthetase. By analyzing a random transposon library, we identified *Escherichia coli* mutants resistant to McC. Transposon insertions were localized to a single locus, *yejABEF*, which encodes components of a putative inner membrane ABC transporter. Analysis of site-specific mutants established that all four components of the transporter are required for McC sensitivity. Since aspartyl-tRNA synthetase in *yej* mutant extracts was fully sensitive to McC, we conclude that *yej* mutations interfere with McC uptake and that YejABEF is the only inner membrane transporter responsible for McC uptake in *E. coli*. Other substrates of YejABEF remain to be identified.

Microcins (Mc) are a class of antibacterial agents produced by *Escherichia coli* and its close relatives (1). Mc are characterized by their relatively small size (<10 kDa) and narrow specificity of antibacterial action. Mc are produced from ribosomally synthesized peptide precursors. Some Mc are heavily modified by dedicated maturation enzymes (2, 6). Genes coding for the maturation enzymes are clustered together with the genes coding for Mc precursors and are often plasmid borne (2). Mc-producing cells are immune to the Mc they produce (16). Cross-immunity analysis identified several Mc immunity classes—A, B, C, etc. (1). An immunity class defines an Mc's structure and, therefore, its mechanism of action. Accordingly, Mc are named by their immunity class.

Analysis of Mc-resistant cells led to the identification of systems responsible for their uptake. For example, Mc E492 (McE492), McH49, and McM utilize the catecholate receptor proteins FepA, Fiu, Cir, and IroN when entering *E. coli* (3). McJ is taken up by ferrichrome receptor FhuA, the TonB-ExbB-ExbD complex, and SbmA (19, 20), while McB seems to require OmpF and OmpR, as well as SbmA, for the uptake (12). The uptake pathway for McC, the subject of this study, is unknown.

McC is a heptapeptide that contains a modified AMP covalently attached to its C terminus through an *N*-acyl phosphoramidate linkage (Fig. 1) (11, 13). The peptide moiety of McC is encoded by 21-bp-long *mccA*, the shortest bacterial gene known (10). Other genes in the *mcc* locus (Fig. 1A) include *mccB*, whose product is homologous to E1 ubiquitin-activating enzymes that covalently attach AMP to proteins; *mccD*, whose product is homologous to protein methylases and likely attaches the propylamine moiety to the phosphate group of McC (T. Kazakov and K. Severinov, unpublished observations); *mccC*, which encodes an export pump that determines the immunity of the producing cell; and *mccE*, a gene whose product appears to be required both for the production of mature McC and immunity towards it (9).

McC is specifically processed inside sensitive cells, and the product of such processing, a nonhydrolyzable aspartyl-adenylate, strongly inhibits translation by preventing the synthesis of aminoacylated $tRNA^{Asp}$ by aspartyl-tRNA synthetase (Asp-RS). Unprocessed McC has no effect on the aminoacylation reaction. On the other hand, processed McC has no effect on McC-sensitive cells. Thus, McC belongs to the so-called Trojan horse type of inhibitors (17): the peptide moiety allows McC to enter sensitive cells, where it must be processed by peptidases to release the inhibitory aminoacyl-nucleotide part of the drug.

The mechanism of McC action implies that at least three distinct types of mutations conferring resistance to the drug can be obtained (Fig. 1B). First, mutations in the target, Asp-RS, could obviously lead to resistance. However, because Asp-RS is an essential enzyme, Asp-RS mutants defective in interaction with processed McC shall also be impaired in the binding of the substrate, aspartyl-adenylate, and are therefore likely to be nonviable. The second type of resistance mutation can affect genes encoding peptidases that perform the suicidal act of McC processing inside the sensitive cell. The *E. coli* genome encodes multiple peptidases with low specificity and redundant functions (15). Since any one of these peptidases might be involved in McC processing, resistance mutations due to defective processing should be difficult to obtain (indeed, our unpublished observations indicate that the simultaneous removal of three peptidases with overlapping specificities is required to block McC processing in *E. coli* extracts [T. Kazakov and K. Severinov]). The third type of resistance mutation could decrease McC uptake or increase its export from the cell. Cells producing McC possess the latter type of resistance due to the function of the MccC transporter (9). In an attempt to identify the *E. coli* transport systems responsible for McC up-

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▽ Published ahead of print on 14 September 2007.

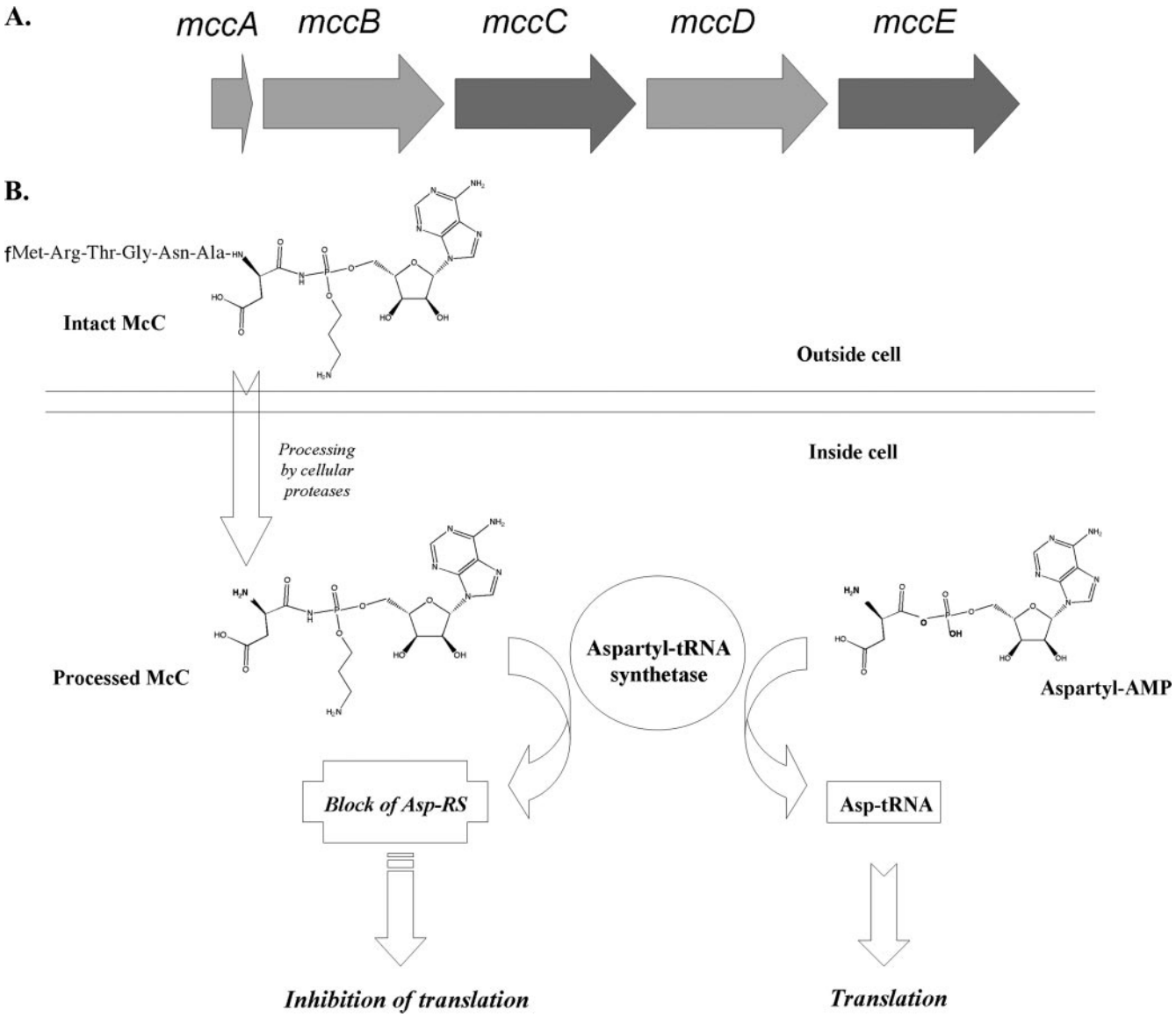

FIG. 1. The mechanism of action of McC. (A) The structure of the *mcc* locus. See text for details. (B) The Trojan-horse mechanism of McC's action is schematically illustrated. Intact McC, a peptide-nucleotide, is taken up by a bacterial cell, where it is processed by peptidases. Processed McC is structurally similar to aspartyl-adenylate, an intermediate of the $tRNA^{Asp}$ aminoacylation reaction catalyzed by Asp-RS. Unlike aspartyl-adenylate, processed McC is nonhydrolyzable. It binds to and inhibits Asp-RS, leading to inhibition of translation.

take, we tested a collection of *E. coli* cells deficient in components of several transport systems known to be involved in the transport of peptides or other Mc in the cell. The strains tested included *fhuA*, *btuB*, *fepA*, *ompA*, *ompC*, *tonB*, *tolA*, *tolB*, *tolC*, *tolR*, *pal*, and *lpp* mutants; none showed any resistance to McC (A. Metlitskaya, unpublished observations). These results implied that McC uses a dedicated, yet-uncharacterized transport system or may even enter the cell by diffusion.

To investigate this issue further, we used a library of McC-sensitive *E. coli* SG289 cells carrying random insertions of the mariner-based transposon TNSC189 (4). The library, which was generously provided by Sean Garrity (Harvard Medical School), is estimated to contain ~90,000 unique transposon insertions (S. Garrity, personal communication) and should therefore contain insertions in most *E. coli* genes that are not essential for survival. To select McC mutants, aliquots of the TNSC189 library were plated on solid LB medium containing 20 μg/ml McC. As a control, aliquots of unmutagenized SG289 cells were used. The plating results showed that the frequency of McC-resistant colonies was much higher for SG289 cells mutagenized with TNSC189 than for control, unmutagenized SG289 (1.5×10^{-3} and 5×10^{-6}, respectively). A TNSC189 insertion makes SG289 cells resistant to kanamycin (4). P1 transductions with 15 randomly selected McC-resistant, kanamycin-resistant clones were performed. The results showed that, for all clones, the McC and kanamycin resistances were cotransduced to an McC-sensitive, kanamycin-sensitive *E. coli* recipient strain (data not shown), indicating that the TNSC189 insertions were responsible for McC resistance.

Cytoplasmic extracts from McC-resistant, kanamycin-resis-

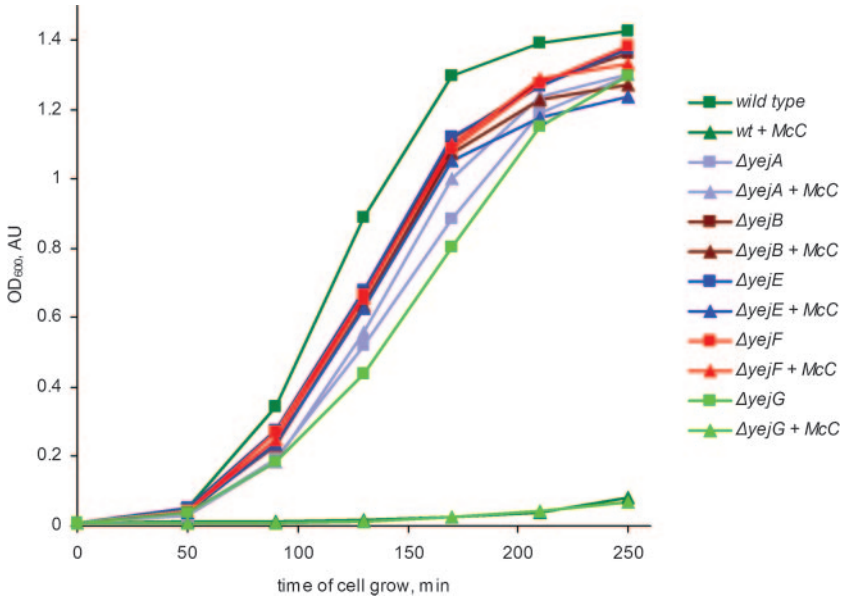

FIG. 2. Cells with mutations in *yejABEF* are McC resistant. The growth of the indicated wild-type and mutant strains in the presence or in the absence of 10 μg/ml of McC is shown. The results shown are representative of those obtained in three independent experiments. OD_{600}, optical density at 600 nm; AU, absorption units; wt, wild type.

tant transductants were prepared, and their ability to process intact McC was tested. Incubation of McC with extracts prepared from transductants or from control McC-sensitive cells resulted in rapid inactivation (processing) of McC, as judged by a dramatic decrease in its ability to produce growth inhibition zones on lawns of sensitive *E. coli* cells (data not shown). On the other hand, extracts containing processed McC were unable to perform $tRNA^{Asp}$ aminoacylation, while extracts without McC were highly active in this reaction, which is catalyzed by Asp-RS (data not shown). We therefore conclude that the McC resistance of transductants is not caused by alterations in the McC processing machinery or the Asp-RS target and is therefore likely due to deficient uptake of the drug. We consider resistance due to increased McC export unlikely since transposon insertions should destroy, not modify, the gene product function.

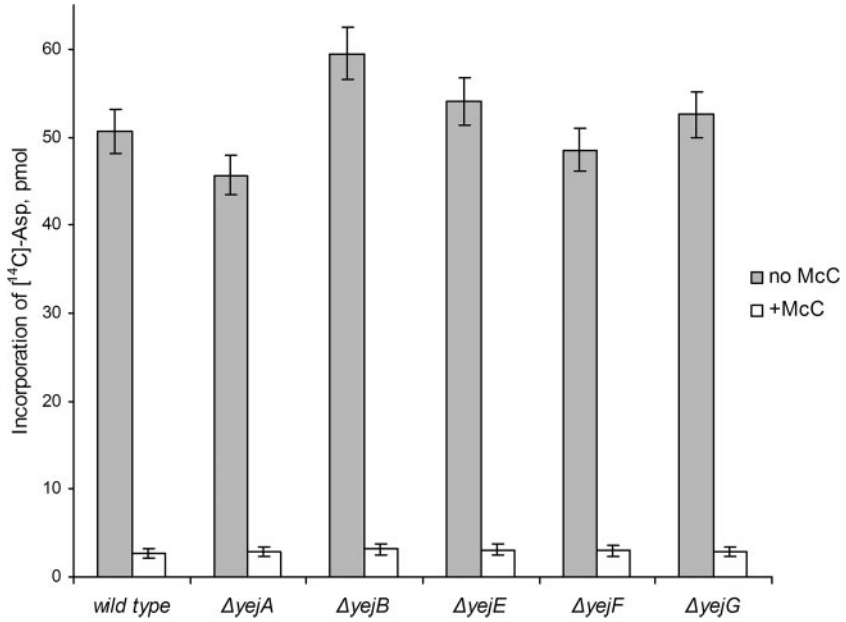

FIG. 3. McC processing and Asp-RS inhibition in extracts of *yejABEF* mutants. S30 extracts of the indicated cells were prepared and incubated with or without 2 μg/ml McC to allow processing, and the $tRNA^{Asp}$ aminoacylation reaction was carried out. The amounts of aminoacylated $tRNA^{Asp}$ (measured as the incorporation of $[C^{14}]$Asp in trichloroacetic acid-precipitable material) are shown. Error bars show standard deviations. See Metlitskaya et al. (14) for experimental details.

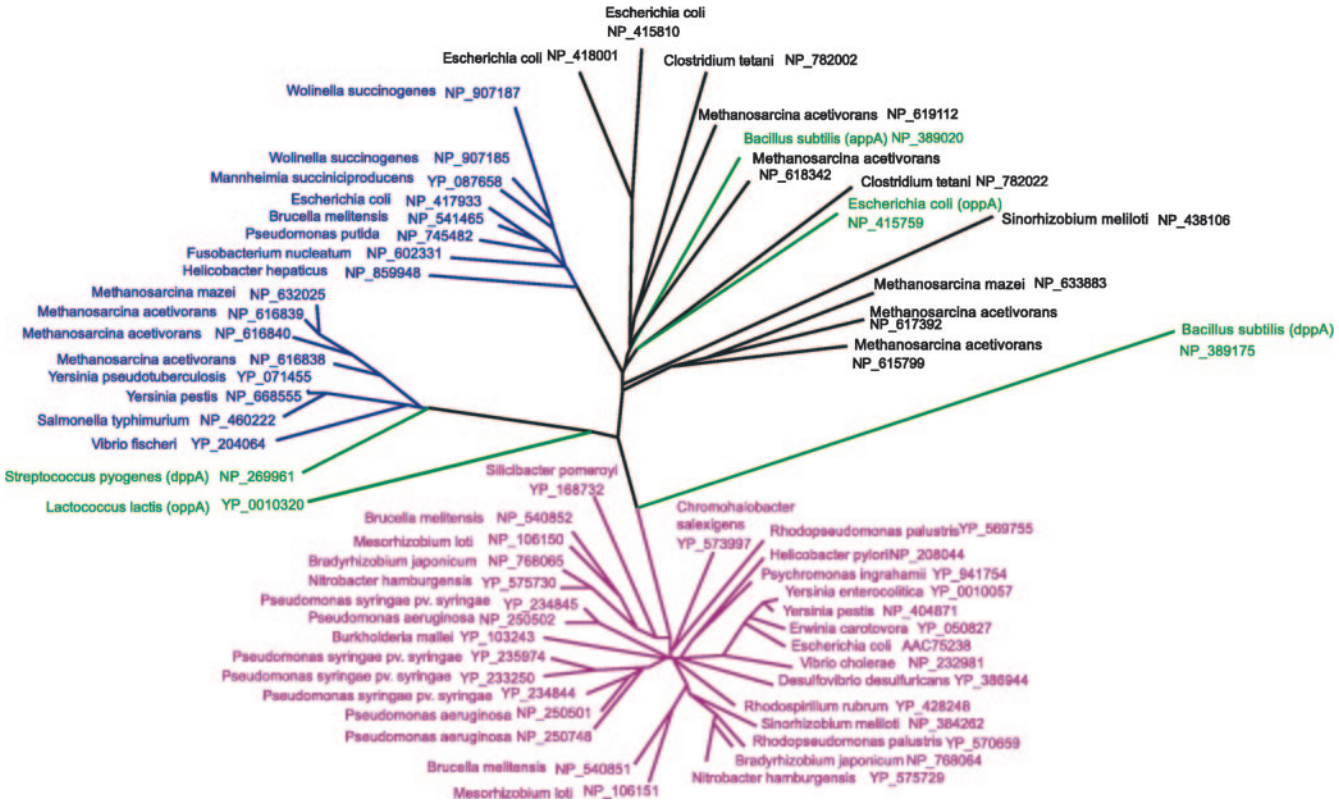

FIG. 4. Phylogenetic tree of periplasmic components of oligopeptide ABC transporting systems. YejA and its close homologs are marked in red, nickel-oligopeptide transporters are marked in blue, experimentally verified proteins are marked in green, and experimentally uncharacterized proteins are in black. Accession numbers of proteins from NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/) are indicated. The tree was created using the NEIGHBOR program from the PHYLIP package (8).

The sites of McC insertion were mapped by using RATE (7), a protocol that involves one-primer PCR amplification of an area at the transposon-chromosomal DNA junction from genomic DNA, followed by sequencing of amplified fragments. In 11 clones, three different insertions in *yejA*, a gene coding for a periplasmic receptor of an ABC transporter of an unknown function (annotated as a putative peptide-Ni transporter), were observed. Three clones contained different insertions in neighboring *yejB*, and one clone had an insertion in the *yejF* gene.

The *yej* cluster of *E. coli* consists of four genes (*yejABEF*) transcribed in one direction and likely forming an operon and a downstream gene, *yejG*, transcribed in an opposite direction. The products of *yejABEF* have easily recognizable similarities to periplasmic receptors (YejA), two transmembrane components (YejB and YejE), and ATPase (YejF) of known inner membrane ABC transporters. The product of *yejG* has no similarities to genes with known functions in publicly available databases and appears to have been given its name based only on the proximity to the *yej* cluster.

The results presented above argue strongly that the Yej transporter is responsible for McC uptake in the cell. To prove this notion directly, we created cells carrying deletions of each of the individual open reading frames of the *yej* locus (5). All mutant cells were viable and showed no visible anomalies in growth on rich medium at 37°C. Five *yej* mutants, along with the parental control *E. coli* BW28357 cells, were tested for their ability to grow in the presence of McC. The results, presented in Fig. 2, showed that deletion of any one of the *yejABEF* genes led to full McC resistance. In contrast, cells lacking *yejG* were as sensitive to McC as the wild-type control cells. Extracts prepared from the *yej* mutant cells processed McC, leading to efficient inhibition of $tRNA^{Asp}$ aminoacylation (Fig. 3). We therefore conclude that an ABC transporter formed by the products of *yejABEF* genes is required for McC uptake in *E. coli*; in fact, YejABEF is the only inner membrane transporter that is required for McC uptake under the conditions used here (rich medium). We also conclude that the *yejG* gene and its product have no functional relationship to the *yejABEF* genes and their products.

The cellular function of the YejABEF transporter is unknown. We tested cells lacking the individual genes from the *yejABEF* cluster for sensitivity/resistance to several antibiotics. No differences from the wild-type control were observed. Natural (i.e., McC-unrelated) substrates of the YejABEF transporter that may point to its cellular function remain to be established.

We used a bioinformatics approach to search for Yej homologues in other bacteria and to look for putative regulatory sites in the *yejABEF* locus that may allow the prediction of its substrates. Phylogenetic analysis revealed conserved operons similar to *yejABEF* in 126 bacterial species belonging to alpha-, beta-, gamma-, delta-, and epsilonproteobacteria. Fifteen species contain two paralogous *yejABEF*-like operons. In some

genomes (including all completed *Pseudomonas* genomes), two paralogous tandem genes encoding the periplasmic component of the transporter were found.

As some gammaproteobacterial genomes lack operons homologous to *yejABEF*, we looked for the presence of genes that neighbor the *yejABEF* locus in *E. coli*. In the genome of *Photorhabdus luminescens*, a close relative of *Yersinia*, deletion of the whole operon, as well as one of the neighboring genes, *rtn*, was evident, suggesting that the locus was lost. *P. luminescens* is resistant to McC, while its extracts efficiently process intact McC, and the processed McC inhibits the $tRNA^{Asp}$ aminoacylation reaction (data not shown). In *Vibrio* spp., which also lack the *yejABEF* operon, the upstream and downstream neighbors of *yejABEF* from *E. coli* are scattered throughout the genome, and so no inferences can be made about the fate of *yejABEF* in these organisms.

We compared the YejA protein and its close homologues with periplasmic components of other oligopeptide ABC transporters (Fig. 4). The YejA orthologs and close paralogs form a distinct branch on the phylogenetic tree and are well-separated from all known dipeptide or oligopeptide transporters. Similar trees were obtained for other components of the YejABEF transporter (data not shown). Despite its annotation, the Yej transport system has little similarity to known nickel transporters from the nickel/peptide/opine transporter family (18). No NikR-binding motifs were found upstream of the *yejABEF*-like operons (data not shown), supporting the notion that Yej is not involved in nickel transport. Finally, no known or shared conserved regulatory motifs upstream of proteobacterial *yejABEF* orthologs could be identified, possibly suggesting that these transporters perform different functions in different proteobacteria. Indeed, the presence of a *yejABEF*-like operon could not be regarded as a predictor of McC sensitivity, as would be expected if the YejABEF transporter were universally involved in McC transport. For example, *Pseudomonas aeruginosa*, which contains two *yejABEF* homologues, is McC resistant (our unpublished observations). However, McC is efficiently processed in *P. aeruginosa* extracts and its Asp-RS is readily inhibited by processed McC (data not shown). Thus, the resistance of *P. aeruginosa* to McC may be due to the fact that its Yej homologs do not recognize McC or are not expressed under laboratory conditions. Alternative possibilities, such as a lack of permeation through the outer membrane or McC degradation before it reaches the transporter, are also possible.

We are indebted to Sean Garrity for providing us with the transposon library and for advice on RATE and to Alexander Mironov for help with P1 transductions.

This work was supported by a Burroughs Wellcome career award, a Russian Academy of Sciences presidium program in molecular and cellular biology new groups grant, an NIH/NIAID-Lipkin U54 AI057158 Northeastern Biodefense Center grant, a Rutgers University technology commercialization fund grant (to K.S.), Russian Foundation for Basic Research grant 06-04-48865 (to A.M.), and Howard Hughes Medical Institute grant 55000309 and a Russian Academy of Sciences program in molecular and cellular biology grant to M. S. Gelfand.

ADDENDUM IN PROOF

It has come to our attention that *S. enterica* serovar Typhimurium with genetic lesions in the Yej transporter induces a superior $CD8^+$ T-cell response due to increased peptide presentation on MHC-1 class molecules (U. Qimron, N. Madar, H. W. Mittrucker, A. Zilka, I. Yousef, N. Bloushtain, S. H. Kaufmann, I. Rosenshine, R. N. Apte, and A. Porgador, Cell. Microbiol. **6**:1057–1070, 2004), suggesting a role for Yej in oligopeptide transport in this organism.

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