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A *Corynebacterium glutamicum* Gene Conferring Multidrug Resistance in the Heterologous Host *Escherichia coli*

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A chromosomal DNA fragment from the erythromycin-sensitive bacterium *Corynebacterium glutamicum* ATCC 13032 was shown to mediate resistance against erythromycin, tetracycline, puromycin, and bleomycin in *Escherichia coli*. Multicopy cloning of the fragment did not cause a resistance phenotype in *C. glutamicum*. The corresponding gene encodes a hydrophobic protein with 12 potential transmembrane-spanning α-helical segments showing similarity to drug-H^+ antiporters.

Prokaryotes have developed mechanisms to protect themselves against antimicrobial agents (5). Some of these mechanisms are specific for particular toxins, while others confer resistance to a spectrum of different drugs, called multidrug resistance (MDR). The first and most extensively studied example of MDR concerns the eukaryotic P glycoprotein from mammalian cells (3). In recent years, several prokaryotic MDRs which are located in the cytoplasmic membrane and which extrude toxic agents from the cell have been described (9). These transporters belong to four distinct families according to their size, membrane topology, and energy coupling (9).

In this report, we present the isolation of a gene from *Corynebacterium glutamicum* conferring resistance against different drugs to *Escherichia coli. C. glutamicum* is a nonpathogenic, gram-positive microorganism mainly used for the fermentative production of amino acids (10). It is a member of the CMN group comprising the genera *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus* (1). The presence of a gene in *C. glutamicum* conferring resistance to several antibiotics mentioned below was not expected, since *C. glutamicum* itself is sensitive to these drugs.

Cloning of an erythromycin resistance DNA fragment from *C. glutamicum* into *E. coli*. We isolated an erythromycin resistance (Em^r) DNA fragment in the course of rescuing chromosomal transposon insertions from *C. glutamicum* mutant strains (8), which were generated by means of the Em^r transposon Tn*5432* (16). Chromosomal *Eco*RI fragments from Tn*5432*-carrying *C. glutamicum* strains were ligated to *Eco*RI-digested pUC9 (19) DNA, and the ligation mix was electroporated into *E. coli* DH5 MCR (4) according to standard procedures (15). We selected for Em^r on LBG agar (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose) containing 250 μg of erythromycin ml^{-1}. From different *C. glutamicum* mutant strains, however, an identical 4.7-kb DNA fragment was cloned, which displayed a restriction enzyme pattern (Fig. 1A) different from that of Tn*5432* DNA. Subsequent hybridization analysis (data not shown) revealed that this fragment originated from the chromosome of *C. glutamicum*. One of the plasmids, termed pWJ80 (Fig. 1A), was used for further analysis. The corresponding gene was termed *cmr* (corynebacterial multidrug resistance), since it was found to mediate resistance to several structurally unrelated antibiotics (see below).

Determination and analysis of the DNA sequence of the *cmr* gene. The DNA sequence of the *cmr* gene was determined in order to obtain more information about the underlying molecular reaction. We constructed several plasmid derivatives by subcloning the *C. glutamicum* DNA insert of pWJ80 and tested their ability to mediate Em^r. The *cmr* gene was confined to a 2.5-kb *Eco*RI/*Sph*I fragment and was found to cause resistance independent of the orientation of the cloned fragments, indicating expression of *cmr* in *E. coli* from its own promoter (data not shown). This DNA fragment was sequenced as described previously (7) and was found to carry one large open reading frame coding for a protein of 459 amino acids (Fig. 1B), which evidently was responsible for mediating the resistance phenotype. The hydropathic profile of the protein displayed high hydrophobicity (data not shown). Prediction of its topology by the TOP-PRED computer program developed by von Heijne (20), which takes into account not only the hydrophobicity analysis but also the rule that positively charged residues cluster on the cytoplasmic face of a membrane protein, led to the prediction of 12 transmembrane-spanning α-helical segments (Fig. 1B). The protein is proposed to be embedded in the cytoplasmic membrane, with the N- and C-terminal ends as well as the central hydrophilic region being located inside the cell. This structure is common for transport proteins belonging to the major facilitator family, whose members mainly act as drug/H^+ antiporters or sugar/H^+ symporters (12). Database searches showed weak amino acid sequence similarities to several drug exporters, such as Tn*10* TetA (13) and Pur8 from *Streptomyces alboniger* (17), which confer resistance to tetracycline and puromycin, respectively.

Assessment of the resistance range mediated by the *cmr* gene in *E. coli*. Sequence similarities of Cmr to different drug extrusion proteins inspired us to check whether resistance to antibiotics other than erythromycin was also mediated. In a systematic screening, we analyzed the resistance spectrum due to the gene. *E. coli* cells carrying plasmid pWJ80 or pUC9, respectively, were spread on LBG agar, and small filter disks supplemented with antibiotics belonging to different families (2) were placed onto the plates. The sizes of the growth inhibition zones were compared after overnight incubation at 37°C. The disk test showed increased resistance to erythromycin, tylosin, rifampin, tetracycline, D-cycloserine, bleomycin, puromycin, and novobiocin, but not to gentamicin, kanamycin, neomycin, streptomycin, flavomycin, chlorotetracycline, mitomycin, nalidixic acid, norfloxacin, cefotaxime, polymyxin B, chloramphenicol, and trimethoprim. The resistance levels were determined more precisely by dilution of overnight cultures 1:100 in LBG liquid medium supplemented with antibiotics in different concentrations and measuring the

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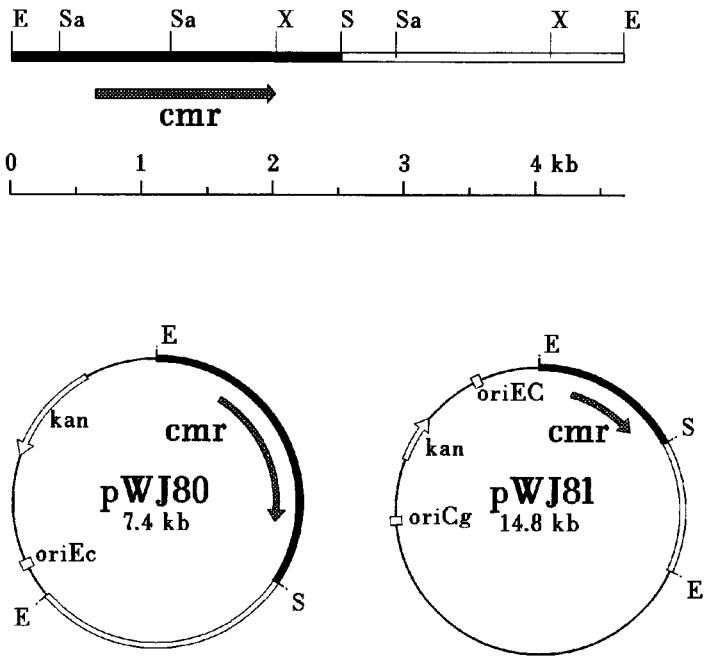
(A)



(B)

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   1 GAATTCCCCAGCACAATTTGTCCATGATCACCGTGGCAGCCGCAGCGCCCGACAGCGCCCATTTGGTTTTGTGCTGTTGGAAAAGCTCCGGTAGCTCAGCGATGCCGCGTGGATTGAGTT

 121 TCACAAACTGCGCATCCTGGCGATGTGTGATGCCCTGAAGCGTTCCGGCTTCAGAGACAAGGGTGAGCGGATCGTTGATATTCCACCGCTCAAAACGGTGCAACCAAGCGAGAATTTCCT

 241 GGTTGTGGGGATTTTCGGGGACGGAGGCGTCGATAAGCGTGCGCCTGCAGCGGCCGCAAAGGGATCGCAGGCGTGGTGCGCGGCGACCTTAATATCAAGCCCGAGCGCCAACGCGACCTC

 361 ACGCAGCGTCGACAGGGTGGGCTCGCTGGTGCCATCGCCGACCCGCTTCAGGGTCGAGCGCGAGACACCGGAACGCCTGGAGATGCGAGTGGGTTGCTCCGCGGCGAGAGCAATCAATTC

 481 ATCGATCTTCACAACAAACCATGCTAATCATCACGGTTTTTCGCTCAGGCACCGGCCAACGCTTTTCGACGCGCCCCTCCACCTTTTCAGTAGCGTCACGGGCGCCAATCCTGTATTTTT

                                                  RBS       V  S  T  F  H  K  V  L  I  N  T  M  I [S  N  V  T  T  G  F  L  F  F  A  V ]
 601 AGCAGCAGTTTGAGGGTTTTGCTCCCCATCTTTAGGAGACACCCCGTGTCCACGTTTCATAAAGTTCTGATCAACACCATGATCTCCAACGTCACCACTGGATTTCTGTTCTTTGCCGTG

  26 [V  F  W  M  Y  L  S  T  G] N  V  A  L  T  G [I  V  S  G  I  Y  M  G  L  I  A  V  C  S  I  F  F  G  T  V  V] D  H  N  R
 721 GTGTTTTGGATGTATCTTTCCACTGGCAACGTCGCACTGACCGGCATCGTCAGTGGAATTTACATGGGTTTGATCGCCGTTTGTTCCATCTTTTTCGGAACCGTTGTTGATCACAATCGC

  66 K  K  S  V  M  L [F  S  S  V  T  T  L  V  F  Y  C  L  S  A  L  V  W  V  F  W  L] E  E  D  G [L  S  I  G  N  T  A  L  W ]
 841 AAGAAGTCCGTCATGCTGTTTTCCAGCGTCACCACACTCGTGTTTTATTGTCTCAGTGCCCTGGTGTGGGTGTTTTGGCTGGAGGAAGACGGCCTGAGCATCGGAAATACCGCCCTGTGG

 106 [V  F  V  S  F  I  L  I  G  S  I  V] E  H  M  R  N  I  A  L  S  T  V  V  T  L  L  V  P  E  A  E  R  D  K  A  N  G  L  V
 961 GTGTTCGTTTCTTTCATCCTCATCGGATCAATCGTGGAACACATGCGCAACATCGCACTGTCCACCGTGGTCACGCTGTTGGTTCCTGAAGCTGAACGCGACAAAGCAAACGGCCTGGTA

 146 G  A  V [Q  G  V  G  F  L  V  T  S  V  I  A  G  S  A  I  G  F  L  G  M] E  I [T  L  W  I  C  L  G  L  S  L  V  A  L  L ]
1081 GGAGCCGTGCAAGGTGTTGGATTTTTAGTCACCAGCGTCATTGCTGGTTCCGCCATCGGGTCTTGGGCATGGAAATCACCCTGTGGATCTGCCTTGGGCTCTCACTTGTCGCGCTGCTG

 186 [H  L  L  P  I  R  V] D  E  P  E  I  I  T  Q  E  D  A  Q  P  T  V  S  D  D  S  V  P  T  P  T  S  D  L  A  I  V  S  K  G
1201 CACCTGCTGCCGATTCGCGTCGACGAACCGGAAATCATCACCCAAGAAGACGCACAGCCTACTGTTTCTGACGATTCAGTTCCCACACCTACCTCCGATTTGGCGATCGTGTCCAAAGGC

 226 I  D  L  K  G [S  M  K  I  I  L  S  V  P  G  L  L  A  L  V  L  F  A  S  F  N] N  L  I  G  G  V  Y  S  A  L  M  D  P  Y
1321 ATCGACCTAAAAGGATCAATGAAAATCATCCTGAGTGTTCCGGGACTGCTCGCGCTTGTGTTGTTTGCGTCCTTCAACAACCTCATCGGCGGCGTGTACTCCGCACTCATGGACCCTTAC

 266 G  L  E  L  F  S  P  Q [L  W  G  L  L  L  G  L  T  S  L  G  F  I  V  G  G  A  V  I  S] K  T  G  L  G  K [N  P  V  R  T ]
1441 GGCCTGGAACTTTTCAGCCCACAGCTGTGGGGGCTACTGCTTGGACTCACCAGCCTCGGCTTCATCGTTGGTGGTGCTGTGATCTCCAAAACTGGCTTGGGCAAAAACCCTGTGCGCACC

 306 L  L  L  V  N  V  G  V  A  F  V  G  M  L  F  A  I  R [E  W  W  W  L  Y  I  L  G  I  F  I  F  M  A  I  T  P  A  A  E ] A
1561 TTGCTGCTGGTTAATGTTGGTGTGGCTTTTGTTGGCATGTTATTTGCCATTCGCGAATGGTGGTGGCTCTACATCCTGGGCATTTTCATCTTCATGGCTATCACCCCAGCTGCCGAAGCC

 346 [A  E  Q  T  I  L  Q  R  V  V  P  F  R  Q  Q  G] R  V  F  G  L  A  M  A  V  E  M  A  A  N [P  L  S  T  V  I  V  A  I  L ]
1681 GCAGAACAAACCATCCTTCAACGAGTCGTCCCATTCCGCCAACAAGGCCGCGTATTTGGACTAGCCATGGCAGTGGAAATGGCAGCCAACCCGCTCTCCACAGTGATCGTGGCGATTTTG

 386 [A  E  A  Y  L  I  P  W  M  A  G] P  G  A  D  T  I  W  G  V  I  L  G  E  G  K  A  R  G [M  A  L  M  F  L  A  S  G  A  I ]
1801 GCCGAAGCCTACCTCATTCCATGGATGGCTGGCCCCGGCGCGGACACCATCTGGGGCGTGATCCTCGGCGAGGGTAAAGCTCGCGGCATGGCACTGATGTTCCTCGCATCAGGTGCCATC

 426 [M  L  V  V  V  L  L  A  F  M] S  R  S  Y  R  K  L  S  Q  Y  Y  A  T  T  S  Q  D  I  A  G  A  A  E  K  *  459 amino acids
1921 ATGTTGGTTGTCGTGCTGTTGGCATTCATGTCGAGGTCCTACCGGAAACTCAGCCAGTACTACGCCACCACCAGCCAAGACATTGCGGGAGCTGCTGAGAAGTAAGTGCTCTAGACCGTT

2041 GTTTGATTGGCTTTTCTTGCCTGTTGAACGAGGGAAAGAAAAACAAACAGTCCTGAAGCTACACCCAAAGAAGCAGCATAAATTGGTTCCGCAGGAACCCATTGGCAACAAAACACAAGA

2161 TAATCATTGCGCCAACTTGCCAAACCGGGGCCCGCTTTTTCCAAAATCGAACTGTGGAGAATTTATTAATCCCCCATGACAAGGCAACAAGGAGGCCGATGAAAGCTATGGCTAAAAGAA

2281 CTTGATTCAGAGTTAGCGAGTAAATGAACAATCCAAAAAAACATAGAAGCGATAAAACTCTGCCAGTACATATTCACCTTGAGAGGCTTTGGGTATCGGTAGTCAACTGATACAGAGGGA

2401 TCTATTAAGTACATTTAAGAGTACTTCACTTTGAATCGTTGTTCTGGGATTCTCTCAACCCCGGGCCTAAAACGTCAGCTCTCTACAATTAGGCGCTCTATAACGCCCTGAAAATTACCC

2521 CCTGAGCATGC 2531 bps
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FIG. 1. (A) Restriction maps of the 4.7-kb *Eco*RI fragment (box) harboring the *cmr* gene, the *E. coli* plasmid pWJ80, and the *E. coli*/*C. glutamicum* shuttle vector pWJ81. The sequenced region (solid box) and the origins for vegetative replication in *E. coli* (oriEc) and *C. glutamicum* (oriCg) are indicated. E, *Eco*RI; Sa, *Sal*I; S, *Sph*I; X, *Xba*I. (B) Nucleotide and deduced amino acid sequence of the 2.5-kb *Eco*RI/*Sph*I fragment encoding Cmr. The restriction enzyme sites, the ribosome binding site (RBS), of the *cmr* gene, and 12 transmembrane-spanning segments (boxes), which were identified as "certain" by the TOP-PRED program (20), are marked.

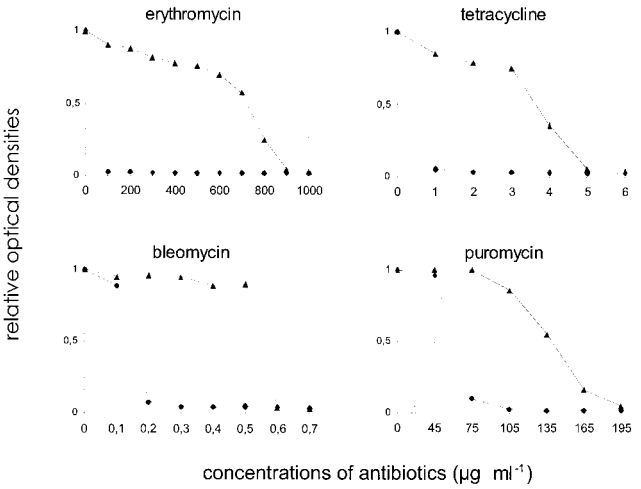

FIG. 2. Antibiotic resistance levels determined in liquid medium of *E. coli* carrying the *cmr* gene on a multicopy plasmid. An *E. coli* strain containing plasmid pWJ80, carrying the *cmr* gene (▲), was compared to an *E. coli* strain containing the plasmid pUC9 (●), which was used as a control strain. The cells were grown in LBG with different concentrations of the drugs (in micrograms per milliliter). Growth is specified as optical density relative to that of a strain without addition of the drug which was set to 1.

optical densities at 580 nm after an incubation for 20 h at 37°C. *E. coli* cells harboring plasmid pWJ80 were found to be clearly resistant to erythromycin, tetracycline, puromycin, and bleomycin (Fig. 2) and displayed slightly increased tolerance to rifampin, tylosin, novobiocin, and D-cycloserine. Since these drugs belong to different classes comprising structurally unrelated compounds (2), it was concluded that the *C. glutamicum* gene mediates multidrug resistance, and the gene was therefore termed *cmr.*

Analysis of drug resistance of *C. glutamicum* cells carrying the *cmr* gene in multiple copies. To test the *C. glutamicum* gene in its original host, we cloned the isolated *C. glutamicum* DNA into the *E. coli*/*C. glutamicum* shuttle vector pECM2 (6), which replicates in both organisms, and transferred the resulting plasmid pWJ81 (Fig. 1A) as well as vector pECM2 to *C. glutamicum*. Plasmid pWJ81 confers the same antibiotic resistance phenotype to *E. coli* cells as pWJ80 (data not shown). However, *C. glutamicum* cells harboring none or either one of the two plasmids were found to be sensitive to 2 μg of erythromycin ml^{-1} added to LBG liquid medium. Comparison of tetracycline, puromycin, and bleomycin resistance levels of *C. glutamicum* harboring plasmid pWJ81 with cells carrying the cloning vector also did not reveal any differences (data not shown). These results imply that the cloned fragment is not sufficient to cause resistance, at least in its original host *C. glutamicum.*

Concluding remarks. In this work, the existence of a multidrug resistance gene originating from a corynebacterial strain has been reported for the first time. The topology of the corresponding protein suggested that it is inserted into the cytoplasmic membrane. Twelve potential transmembrane segments and sequence similarities to other drug resistance proteins imply that Cmr acts as a pump, removing different antibiotic compounds from the cytoplasm of the cell. To our surprise, the *cmr* gene only conferred a resistance phenotype to *E. coli*, but not to *C. glutamicum*. This lack of function in *C. glutamicum* could be the result of a lower expression rate, possibly due to the presence of a repressor preventing *cmr* expression, which is missing when the gene is cloned in the heterologous host *E. coli*. A comparable repression was reported for the *E. coli* MDR pump EmrAB, which is negatively regulated by the *emrR* gene product (11). Moreover, *E. coli* is surrounded by an outer membrane that functions as an effective barrier, while the cell wall structure of the gram-positive bacterium *C. glutamicum* offers only little resistance to the entry of antibiotics (14). The extrusion of drugs by the action of Cmr may be sufficient for *E. coli* but too weak for *C. glutamicum* to mediate resistance, because antibiotics can reenter the *C. glutamicum* cells easier. A weak point of the latter hypothesis is the fact that the TetA-mediated resistance level of *E. coli* to tetracycline is not affected by the efficiency of the outer membrane barrier (18). Nevertheless, the reason for the absence of a resistance phenotype in *C. glutamicum* and the true function of the *cmr* gene in this host still have to be investigated in detail.

Nucleotide sequence accession number. The nucleotide sequence of the *C. glutamicum cmr* gene has been deposited in GenBank under accession number U43535.

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