Plasmid-borne macrolide resistance in Micrococcus luteus

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A plasmid designated pMEC2 which confers resistance to erythromycin, other
macrolides, and lincomycin was detected in Micrococcus luteus strain MAW843
isolated from human skin. Curing of this approximately 4·2 kb plasmid from
the host organism resulted in erythromycin sensitivity of the strain.

Introduction of pMEC2 into a different M. luteus strain conferred erythromycin
resistance upon this strain. Macrolide resistance in M. luteus MAW843 was an
inducible trait. Induction occurred at subinhibitory erythromycin
concentrations of about 0·02–0·05 µg ml−1. Erythromycin and oleandomycin
were inducers, while spiramycin and tylosin exerted no significant inducer
properties. With heterologous expression experiments in Corynebacterium
glutamicum, using hybrid plasmid constructs and deletion derivatives thereof,
it was possible to narrow down the location of the plasmid-borne
erythromycin-resistance determinant to a region of about 1·8 kb of pMEC2.

Sequence analysis of the genetic determinant, designated erm(36), identified
an ORF putatively encoding a 281-residue protein with similarity to 23S rRNA
adenine N6-methyltransferases. erm(36) was most related (about 52–54% identity) to erythromycin-resistance proteins found in high-G+C Gram-positive
bacteria, including the (opportunistic) pathogenic corynebacteria
Corynebacterium jeikeium, C. striatum, C. diphtheriae and Propionibacterium
acnes. This is believed to be the first report of a plasmid-borne, inducible
antibiotic resistance in micrococci. The possible role of non-pathogenic,
saprophytic micrococci bearing antibiotic-resistance genes in the spreading of
these determinants is discussed.

Keywords: erythromycin resistance, erm(36), induction, curing

INTRODUCTION

Phylogenetically, Micrococcus species belong to the broad group of Gram-positive bacteria with a high
G+C content of their DNA. In a new hierarchical
classification system for the class Actinobacteria they
have been allocated to the family Micrococcaceae within
the order Actinomycetales (Stackebrandt et al., 1997).
Micrococci, e.g. Micrococcus luteus and Micrococcus
lyiae, are often isolated from the skin of mammals,
where they appear to be saprophytes. Micrococcus
species are distinguished by various criteria from the
second major group of cocci frequently isolated from
skin, i.e. the staphylococci (Kocur et al., 1992).

Despite their role as common skin inhabitants, little is
known about the occurrence and the mechanism of
action of antibiotic-resistance determinants in micro-
cocci. However, recent work has demonstrated that
significant numbers of erythromycin-resistant M. luteus
strains can be isolated from human skin (Eady et al.,
2000). We have observed that a plasmid-bearing M. luteus
strain designated MAW843 was resistant to
erthyromycin and other macrolide antibiotics (unpub-
lished data). Plasmids with sizes ranging from 1 to about
90 MDa have been detected in micrococci before, but
information regarding their possible functions is very
scarce (Mathis & Kloos, 1969). Therefore, we decided
to investigate if the antibiotic-resistance phenotype of
M. luteus MAW843 was linked to its plasmid. Also, it
was of interest to compare the resistance gene of this
strain with previously characterized erythromycin-re-
sistance determinants. Furthermore, since no cloning
systems have been established for *Micrococcus*, an indigenous *M. luteus* antibiotic-resistance plasmid may be useful for genetic engineering purposes.

**METHODS**

**Bacterial strains and media.** The bacterial strains and plasmids used are listed in Table 1. *Micrococcus luteus* strain MAW843 was isolated during a survey of the microbial population on the skin of a female person treated with clindamycin for acne. For transformation experiments, *M. luteus* ATCC 27141 was used. *M. luteus* strains were propagated at 30 °C using P medium (0.5% peptone, 0.5% sodium chloride, 0.1% glucose) or LB medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride; pH 7.2). Where appropriate, the media were supplemented with agar (12 g l⁻¹) and/or antibiotics as described in the text. *Corynebacterium glutamicum* strains containing hybrid plasmids were grown in LB medium supplemented with 20 µg kanamycin ml⁻¹.

**Isolation of plasmid DNA from *M. luteus* cells.** For large-scale plasmid DNA isolation, cells of *M. luteus* from a total of 2 litres of P-broth culture were harvested and suspended in 40 ml 50 mM Tris/HCl pH 7, 10 mM EDTA. Cell lysis was achieved by treatment with lysozyme (15 min at 37 °C at a final concentration of 5 mg ml⁻¹) and subsequent addition of 3 ml 0.5 M EDTA pH 8 and 5 ml 10% SDS. The lysate was mixed with 10 ml 5% sodium chloride, incubated at 0 °C for 120 min, and cleared by centrifugation (23 500 g, 30 min, 4 °C). The DNA was precipitated with 0.7 vol. 2-propanol, collected by centrifugation, redissolved in 10 mM Tris/HCl pH 8, 1 mM EDTA (TE), and purified further via caesium chloride/ethidium bromide equilibrium gradient centrifugation (Sambrook et al., 1989). Small amounts of plasmid DNA were isolated from *M. luteus* strains by a rapid boiling method. Cells of a 3–5 ml culture were suspended in 100 µl 40 mM Tris/acetate buffer pH 8, 2 mM EDTA and mixed with 150 µl 1 M sorbitol and 30 µl 100 mg ml⁻¹ lysozyme solution. After 15 min at 37 °C, 25 µl 0.5 M EDTA pH 8 and 50 µl 10% SDS were added. The suspension was boiled for 2 min, cooled on ice, and cleared by centrifugation for 15 min in a table-top centrifuge. The lysate was extracted once with phenol/chloroform (1:1, v/v) and once with chloroform. The aqueous phase was transferred to a fresh tube and the DNA was precipitated by the addition of 0.1 vol. 3 M sodium acetate and 1 vol. 2-propanol (20 °C, 15 min). After centrifugation for 15 min, the DNA was dissolved in TE buffer, reprecipitated (this time with 2 vols ethanol at −20 °C for 2 h), and finally dissolved in TE buffer.

**DNA analysis and modification, and construction of hybrid vectors.** Restriction endonucleases and other modifying enzymes were purchased from Pharmacia, Boehringer or New England Biolabs, and used as recommended by the suppliers. In general, DNA sequencing and other modifications were carried out according to standard procedures (Sambrook et al., 1989). Computer analysis of nucleotide sequences and the deduced amino acid sequences was performed with the

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**Table 1. Strains and plasmids**

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<th>Strain</th>
<th>Characteristics</th>
<th>Plasmid</th>
<th>Reference/source</th>
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<tr>
<td><em>Micrococcus luteus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAW843</td>
<td></td>
<td>pMEC2</td>
<td>This work</td>
</tr>
<tr>
<td>MAW843-46,1</td>
<td>MAW843 cured of pMEC2, Em⁺</td>
<td>None</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 27141</td>
<td>Em⁺, purine auxotrophic</td>
<td>None</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATCC 27141-5/1</td>
<td>ATCC 27141 transformant, Em⁺</td>
<td>pMEC2</td>
<td>This work</td>
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<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E12</td>
<td>Restriction-deficient mutant of ATCC 13059 derivative AS019</td>
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<td>Follettie &amp; Sinskey (1986)</td>
</tr>
<tr>
<td>R163</td>
<td>Restriction-deficient mutant of ATCC 13059 derivative AS019</td>
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<td>Liebl &amp; Schein (1990)</td>
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</table>

<table>
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<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>Characteristics</th>
<th>Replication*</th>
<th>Markers†</th>
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<tr>
<td>pMEC2</td>
<td>4.2</td>
<td></td>
<td><em>M</em>.I.</td>
<td>Em⁺ (M*.I.*, C.g.)</td>
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<td>pWST1</td>
<td>10.4</td>
<td><em>C. glutamicum</em>/<em>E. coli</em> shuttle vector</td>
<td>C.g., E.c.</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.)</td>
<td>Liebl et al. (1989b)</td>
</tr>
<tr>
<td>pWST1B</td>
<td>10.4</td>
<td><em>C. glutamicum</em>/<em>E. coli</em> shuttle vector</td>
<td>C.g., E.c.</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.)</td>
<td>This work</td>
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<td>pWST3B</td>
<td>6.9</td>
<td>Derivative of pWST1B lacking a 3.5 kb non-essential region</td>
<td>C.g., E.c.</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.)</td>
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<td>pWMC37</td>
<td>11.3</td>
<td>pMEC2-pWST3B hybrid plasmid</td>
<td><em>C</em>.g., E.c., (M*.I.*, C.g.,)</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.), Em⁺ (M*.I.*, C.g.)</td>
<td>This work</td>
</tr>
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<td>pWMC65</td>
<td>11.3</td>
<td>pMEC2-pWST3B hybrid plasmid</td>
<td><em>C</em>.g., E.c., (M*.I.*, C.g.,)</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.), Em⁺ (M*.I.*, C.g.)</td>
<td>This work</td>
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<td>pWMC75</td>
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<td>pMEC2-pWST3B hybrid plasmid</td>
<td><em>C</em>.g., E.c., (M*.I.*, C.g.,)</td>
<td>Ap⁺ (E.c.), Km⁺ (C.g.), Em⁺ (M*.I.*, C.g.)</td>
<td>This work</td>
</tr>
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</table>

*Abbreviations: C.g., *Corynebacterium glutamicum*; E.c., *Escherichia coli*; M.I., *Micrococcus luteus*.

† The initials in parentheses indicate the species (see previous footnote) that we presently know to be capable of expression of the markers.

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programs FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al., 1990). Pairwise alignments for the calculation of protein sequence similarities were created with the program GAP included in the University of Wisconsin Genetics Computer Group (GCG) software package for UNIX (Devereux et al., 1984). Multiple amino acid sequence comparison was done with CLUSTAL W (Thompson et al., 1994).

The *C. glutamicum*/E. coli shuttle vector pWST3B was constructed from pWST1 (Liebl et al., 1989b). First, one BamHI site proximal to the multiple cloning site of pWST1 was removed by BamHI partial digestion, Klenow DNA polymerase fill-in, and religation, yielding pWST1B. Then, pWST1B was converted into pWST3B by deletion of about 3.4 kb of non-essential DNA. This vector was used for the construction of hybrid vectors by fusion with pMEC2.

**Antibiotic susceptibility tests.** The MICs of antibiotics were determined by inoculation of 2 ml aliquots of serial twofold dilutions of antibiotics in P broth (final concentrations from 0.06 to 1024 \(\mu\)g ml\(^{-1}\)) with \(2 \times 10^8\) cells. The cultures were incubated with shaking at 30 °C and monitored for 3 days. For agar diffusion tests, cells of *M. luteus* strains were evenly spread onto P or LB agar plates either by a top agar technique or via plating in order to obtain a confluent lawn of bacterial growth. Then, filter disks containing defined amounts of various antibiotics were placed on the surface of the plates and incubation was carried out at 30 °C for 2–5 days. For the detection of inducer properties of antibiotics, filter disks soaked with possible inducer antibiotics were placed in the centre of plates seeded with *M. luteus* cells. After incubation at 32 °C for 3 h, disks soaked with other antibiotics were arranged around the central disk, and incubation was continued for 2–3 days. The distortion of the growth inhibition halo around a satellite disk in the direction facing the central disk was an indication that the latter disk contained an inducing antibiotic.

**Plasmid curing, transformation, and spiramycin filter strip test.** A fresh overnight culture of the plasmid-bearing *M. luteus* strain MAW843 was diluted 1:5000 in several tubes with P-broth containing ethidium bromide at various concentrations (0.3–50 \(\mu\)g ml\(^{-1}\)). The tubes were incubated on a rotary shaker at 30 °C for about 20 h. An aliquot was drawn from the culture with the highest ethidium bromide concentration where visible growth had occurred. Appropriate dilutions were plated onto LB agar plates and after growth were replica-plated onto plates supplemented with 2.5 \(\mu\)g erythromycin ml\(^{-1}\) in order to screen for erythromycin-sensitive isolates. Transformation of *C. glutamicum* with hybrid plasmids and deletion derivatives was carried out as described before (Liebl et al., 1989a; Yoshihama et al., 1985). Transformsants were selected with kanamycin (20 \(\mu\)g ml\(^{-1}\)).

For electrottransformation experiments with *M. luteus* cells, a method originally developed for *C. glutamicum* (Liebl et al., 1989a) was used except that *M. luteus* was grown in brain heart infusion broth (Difco) instead of LB. In some cases, the washing buffer was modified by substituting Tris for HEPES buffer and/or 10% (v/v) glycerol for 10% sucrose. After electroporation, the cells were diluted 1:10 into P broth supplemented with 0.04 \(\mu\)g erythromycin ml\(^{-1}\), incubated at 30 °C for 3 h, and plated onto P agar plates containing 2 or 5 \(\mu\)g erythromycin ml\(^{-1}\). Unfortunately, after 4–5 days at 30 °C, numerous spontaneous (chromosomal) erythromycin-resistant mutants that did not contain a plasmid grew on the selection plates. In order to screen for true pMEC2 transformants, a simple spiramycin agar diffusion method was developed that allows discrimination between a constitutive resistance phenotype typical for the spontaneous mutants and the inducible resistance phenotype expected for transformants bearing pMEC2. The method was based on the observation that *M. luteus* MAW843 cells induced with erythromycin were resistant to spiramycin, but spiramycin itself does not induce resistance development. Strains to be tested were inoculated as parallel streaks onto the surface of two P agar plates, one of which contained a subinhibitory concentration (0.05 \(\mu\)g ml\(^{-1}\)) of erythromycin. After 4–6 h at 30 °C, a 6 mm wide strip of filter paper soaked with 100 \(\mu\)l of a 1 mg ml\(^{-1}\) spiramycin solution was laid perpendicular to the bacterial streaks on each plate. After 2 days at 30 °C, the strains with the inducible resistance phenotype (pMEC2-bearing strains) revealed a broad zone of growth inhibition next to the antibiotic strip on the plate without erythromycin but not on the other plate with 0.05 \(\mu\)g erythromycin ml\(^{-1}\). With plasmid-free sensitive strains or spontaneously constitutive chromosomal mutants, the growth inhibition zones next to the spiramycin strip or the lack thereof, respectively, were identical on both parallel plates.

**RESULTS**

**Inducible macrolide resistance in *M. luteus* MAW843**

The isolate MAW843 was identified as *M. luteus* by the following criteria (see Kocur et al., 1992): macroscopic colony characteristics, microscopic morphology, and various physiological characteristics (data not shown). The G+C content of strain MAW843 chromosomal DNA was 68.5 mol% (data not shown). The strain was found to be resistant not only to clindamycin, but also to other macrolide and lincosamide antibiotics (Fig. 1). The MICs of erythromycin, oleandomycin and lincomycin for *M. luteus* MAW843 were 512 \(\mu\)g ml\(^{-1}\), >1024 \(\mu\)g ml\(^{-1}\) and 1024 \(\mu\)g ml\(^{-1}\), respectively. Strain MAW843 was susceptible to tylosin (MIC 1 \(\mu\)g ml\(^{-1}\)) but developed increased resistance to this antibiotic in the presence of subinhibitory concentrations of erythromycin (MIC 4 \(\mu\)g ml\(^{-1}\) when grown in the presence of 0.05 \(\mu\)g erythromycin ml\(^{-1}\)). Strain MAW843 was moderately resistant to spiramycin (MIC 64 \(\mu\)g ml\(^{-1}\)), but the MIC increased to 1024 \(\mu\)g ml\(^{-1}\) in the presence of 0.05 \(\mu\)g erythromycin ml\(^{-1}\). These results showed that the resistance phenotype of *M. luteus* strain MAW843 was an inducible trait. This was also observed with an agar diffusion technique on nutrient agar plates seeded with *M. luteus* MAW843 cells (see Methods). In this test, diffusion of an inducing antibiotic from a filter disk on the agar surface caused the distortion of the growth inhibition halo around a neighbouring disk soaked with one of the antibiotics named above (Fig. 2). In this way, erythromycin and oleandomycin, both 14-membered ring macrolides, were identified as potent inducers of macrolide resistance in *M. luteus* MAW843. The 16-membered-ring macrolides tylosin and spiramycin were not inducers.

**Identification of a plasmid associated with erythromycin resistance in *M. luteus* MAW843**

A 4.2 kb plasmid was detected in *M. luteus* strain MAW843 and designated pMEC2. In order to find out if
Fig. 1. Agar diffusion assay for the qualitative detection of antibiotic resistance in *M. luteus* MAW843 and its plasmid-cured derivative MAW843-46,1. Note the reduction of the inhibition zone diameter around the disks with the antibiotics clindamycin (C, 2 µg), lincomycin (L, 2 µg) and spiramycin (S, 100 µg) with strain MAW843 upon growth in the presence of a subinhibitory concentration of erythromycin (central plate) as compared to the plate without erythromycin (left plate). Similar inhibition zone diameters were observed on the MAW843-seeded plates around the disks containing oleandomycin (O, 15 µg), an inducing macrolide antibiotic. Loss of the plasmid pMEC2 in the cured strain MAW843-46,1 resulted in increased sensitivity to macrolide antibiotics, which is reflected by large zones of growth inhibition (right plate). Disks with the non-macrolide antibiotics polymyxin B (P, 300 µg) and thiamphenicol (T, 30 µg) were included on each plate as controls.

Fig. 2. Inducers and non-inducers of macrolide resistance in *M. luteus* strain MAW843. Abbreviations: E, erythromycin; O, oleandomycin; S, spiramycin; T, tylosin. The amount of antibiotic on the central filter disks was 7.5 µg (spiramycin and tylosin) or 30 µg (erythromycin and oleandomycin); the surrounding disks contained 75 µg (spiramycin and tylosin) or 250 µg (erythromycin and oleandomycin).

Fig. 3. Spiramycin filter strip test for the qualitative detection of inducible macrolide resistance. A set of representative *M. luteus* strains, i.e. MAW843 (wild-type containing pMEC2), MAW843-46,1 (cured of pMEC2), ATCC 27141 (wild-type, plasmid-free) and ATCC 27141-5/1 (transformant with pMEC2), were streaked onto two P-agar plates, one of which was supplemented with 0.05 µg erythromycin and challenged with the non-inducing macrolide antibiotic spiramycin as described in Methods by application of a filter paper strip containing 0.5 mg of the antibiotic. Macrolide-sensitive strains (MAW843-46,1 and ATCC 27141) or constitutively resistant chromosomal mutants (not shown) displayed identical zones of growth inhibition or lack thereof, respectively, on both plates, while pMEC2-bearing strains (MAW843 and ATCC 27141-5/1) had no inhibition zones on the plate with a subinhibitory concentration of the inducer erythromycin.
this plasmid was associated with the erythromycin-resistance phenotype of the strain, ethidium-bromide-assisted plasmid curing was carried out as described in Methods. A strain, designated MAW843-46.1 was isolated which lacked a plasmid but was indistinguishable from the parent strain in all other phenotypic traits checked. The cured strain was sensitive to macrolide antibiotics and lincomycin (see Fig. 3). The MIC of erythromycin for strain MAW843-46.1 was 0.5 µg ml⁻¹, in contrast to the MIC of 512 µg ml⁻¹ determined for the parent strain MAW843. A detailed map of restriction endonuclease cleavage sites of pMEC2 is shown in Fig. 4.

Transformation of a plasmid-free M. luteus strain with pMEC2

We attempted to introduce pMEC2 purified from M. luteus MAW843 into a different, plasmid-free M. luteus strain. As a recipient we chose M. luteus ATCC 27141, which can easily be distinguished from strain MAW843 in the available databases were the corresponding

demonstrates that the selected strain was an ATCC 27141 derivative. M. luteus ATCC 27141-5/1 contained a plasmid which according to size and restriction endonuclease analysis was identical to pMEC2 (data not shown).

Construction of hybrid plasmids with pMEC2 and heterologous expression of erythromycin resistance in C. glutamicum

The Micrococcus plasmid pMEC2 was linearized with EcoRV or BclI and ligated with the pWST1B (Liebl et al., 1989a) derivative pWST3B opened with SmaI or BamHI, respectively, thus giving rise to the hybrid plasmids pWMC37, pWMC65, pWMC75 (Fig. 5). The hybrid plasmids and various deletion derivatives thereof (Fig. 5) were transformed into C. glutamicum strain E12. The recombinant strains were found with the aid of the pMEC2-bearing strains with an inducible resistance test is a powerful screening method for the detection of plasmid pMEC2.

The nucleotide sequence of the pMEC2 erythromycin-resistance determinant of pMEC2

The nucleotide sequence of the pMEC2 erythromycin-resistance gene and flanking regions was determined. The sequenced 1163 bp BclI–ClaI fragment of pMEC2 had a G+C content of 64 mol%, which is about 5% lower than the G+C content of M. luteus MAW843 chromosomal DNA (68.5 mol%). An ORF was identified whose 281-residue deduced amino acid sequence was similar to 235 rRNA adenine N⁶-methyltransferases encoded by erm genes of various bacteria (Fig. 6). The closest relatives found among known protein sequences in the available databases were the corresponding
Fig. 5. Construction of hybrid plasmids with pMEC2 and deletion derivatives. Plus symbols indicate the ability of the plasmids to confer increased erythromycin resistance upon the heterologous host bacterium *C. glutamicum*. Abbreviations: amp, ampicillin-resistance gene; kan, kanamycin-resistance gene; ori, origin for replication in *E. coli*; rep, putative *C. glutamicum* replication gene.

Fig. 6. Alignment of amino acid sequences of selected erythromycin-resistance methylases. Abbreviations: ermCd, resistance determinant encoded by *Corynebacterium diphtheriae* plasmid pNG2 (X51472; Serwold-Davis & Groman, 1988; Hodgson et al., 1990), which is identical to *ermX* from Tn5432 found in *Propionibacterium acnes* (AF411029) and nearly identical to *ermCX* from Tn5432 of *Corynebacterium striatum* (gi:709806; formerly *C. xerosis*; Tauch et al., 1995); ermJ, resistance determinant from *Streptomyces venezuelae* (AF079138; Xue et al., 1998); erm(36)Ml, resistance determinant of *Micrococcus luteus* plasmid pMEC2 (this work); ermC, resistance determinant encoded by *ermC* of *Staphylococcus aureus* plasmid pE194 (J01755; Horinouchi & Weisblum, 1982).
enzymes from Corynebacterium diphtheriae (53% identity; encoded by plasmid pNG2; Serwold-Davis & Groman, 1988), Propionibacterium acnes (53% identity; identical amino acid sequence to the C. diphtheriae pNG2-encoded determinant; GenBank accession no. AF411029), Corynebacterium striatum (previously classified as C. xerosis) (53% identity; ermCX gene of the composite transposon Tn5432; almost identical amino acid sequence to the C. diphtheriae pNG2-encoded determinant; Tauch et al., 1995, 2000), Corynebacterium jeikeium (51% identity; chromosomal determinant; Rosato et al., 2001). Further sequences with high similarity to the M. luteus erythromycin-resistance ORF were the macrolide-resistance proteins from other high-G+C Gram-positive bacteria, including various Streptomyces species and Aeromicrobium erythreum (previously classified as Arthrobacter sp.). In agreement with the current classification proposed for erm genes by M. C. Roberts (University of Washington; see http://faculty.washington.edu/marilyn/), the designation erm(36) is proposed for the new erythromycin-resistance determinant from M. luteus plasmid pMEC2.

A small 14-residue ORF and several inverted repeat sequences were detected in the approximately 150 bp sequence region upstream of the erm(36) methylase ORF. These structures may be involved in erm(36) regulation (see Discussion).

**DISCUSSION**

**Analysis of the erythromycin-resistance determinant of plasmid pMEC2 and its regulation**

The occurrence of plasmids with sizes ranging from about 1 to 90 MDa in strains of the genus *Micrococcus* has been described before (Mathis & Kloos, 1969). We have now for the first time been able to unequivocally assign a function to a *M. luteus* plasmid. Four lines of evidence demonstrate the presence of a plasmid-borne macrolide- and lincosamide-resistance determinant in *M. luteus* strain MAW843: (i) ethidium-bromide-assisted curing of the plasmid pMEC2 resulted in an erythromycin-resistant strain; (ii) transformation of the plasmid isolated from strain MAW843 into a different, plasmid-free *M. luteus* strain conferred the resistance phenotype upon this strain; (iii) cloning of pMEC2 or parts thereof into a plasmidborne *E. coli* shuttle vector led to hybrid plasmids which conferred elevated levels of erythromycin resistance upon the heterologous host *C. glutamicum*; (iv) finally, an ORF for a polypeptide similar to known erythromycin-resistance proteins was identified by sequence analysis.

There are three principal mechanisms of erythromycin resistance: (i) modification of the target of the antibiotic (the ribosomal 50S subunit), (ii) expulsion of internalized antibiotic molecules via energy-dependent efflux pumps, or (iii) modification of the antibiotic (Ross et al., 1990; Sutcliffe et al., 1996; see Roberts et al., 1999, for an overview). In the case of the *M. luteus* strain MAW843 studied here, erythromycin resistance was unequivocally shown to be mediated by a new erm gene, *erm(36)*, whose deduced amino acid sequence was most closely related to rRNA methylases from high-G+C Gram-positive bacteria of the genera *Corynebacterium*, *Propionibacterium*, *Aeromicrobium* (formerly classified as *Arthrobacter* sp.) and *Streptomyces*. The mechanisms leading to the resistance phenotype in other erythromycin-resistant strains of *M. luteus* isolated from the skin surface of human patients (Eady et al., 2000; Luna et al., 1999) have not been studied in detail.

The expression of erythromycin-resistance determinants is often regulated. In most cases, such as *Staphylococcus aureus* ermC, control occurs posttranscriptionally by translational attenuation: a constitutively synthesized mRNA takes an inactive conformation in the absence of inducer; in the presence of erythromycin, ribosome stalling during translation of a short ORF in the mRNA 5′-region leads to the rearrangement of secondary structures in this region, which again results in the demasking of the translation initiation sequences of the methylase structural gene (see reviews by Dubnau, 1984; Horinouchi et al., 1983; Weisblum, 1983, 1984). In search of DNA sequences that may be involved in regulation of *erm(36)*, we found the following features in the region upstream of the methylase ORF: (i) inverted repeat sequences that could form mutually exclusive secondary structures, and (ii) a small ORF for a 14-residue peptide that overlaps with the largest inverted repeat (Fig. 7). Thus, ribosomes stalled by erythromycin during translation of this small ORF theoretically could disrupt the secondary structure in this part of the control region and thereby trigger a stem–loop redistribution in the leader region of the *erm(36)* transcript. However, since the putative regulatory region of *erm(36)* is separated from the ribosome-binding sequence and start codon of the methyltransferase ORF by about 40 nucleotides, we do not assume an *ermC*-like classical translational attenuation

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**Fig. 7.** DNA sequence upstream of the *erm(36)* ORF. A possible leader ORF and inverted sequence repeats, which may be involved in the regulation of *erm(36)* induction, are indicated as arrows. The ∆G values (determined with the program FOLDRNA) for the three putative hairpin structures are (from left to right) −21.7, −16.8 and −14.5 kJ mol⁻¹.
for erm(36) induction. We rather prefer the idea of a transcriptional attenuation mechanism. Regulation of macrolide resistance by transcriptional attenuation has been proposed before for ermK of Bacillus licheniformis (Choi et al., 1997) and tlrA of Streptomyces fradiae (Kelemen et al., 1994). Further efforts are necessary to prove this hypothesis and unravel the induction mechanism of erm(36).

Only certain macrolide antibiotics (erythromycin, oleandomycin) were efficient inducers for erm(36) expression, whereas others (the 16-membered-ring macrolides spiramycin and tylosin) did not exert a significant induction effect. Differences in the induction specificity of macrolide-lincosamide-streptogramin B (MLS) resistance genes, i.e. differences in the subsets of MLS antibiotics that induce the expression of the genes, have been observed before (Dubnau, 1984; Mayford & Weisblum, 1990). In the case of ribosome-mediated regulation, where ribosomes themselves act as regulators and are directly involved in the induction process, this phenomenon appears to reflect differences in the antibiotics’ modes of interaction with the ribosome (see Mayford & Weisblum, 1990).

**Heterologous expression of erm(36) and potential of pMEC2 as a cloning vector**

On the basis of the following criteria, plasmid pMEC2 may be suitable for use as a cloning vector for *M. luteus*: (i) it is a small plasmid (4-2 kb) with a moderate copy number; (ii) it carries a genetic marker which can be selected for in *M. luteus*; (iii) a detailed restriction endonuclease map is available; (iv) there are several unique restriction sites that may be useful as insertion loci for foreign DNA fragments. However, it will be necessary to define those regions of the plasmid that are essential for replication and stability.

Interestingly, the pMEC2 erythromycin-resistance determinant was expressed in *C. glutamicum*, which belongs to the same bacterial phylum as the original host *M. luteus*, i.e. the high-G+C Gram-positive bacteria. However, the level of erythromycin resistance reached by *C. glutamicum* transformants bearing hybrid pMEC2-pWST3B plasmids was rather low (resistant to 0.5–1 µg ml⁻¹ on solid media; no growth at 3 µg ml⁻¹; data not shown) as compared with *M. luteus* strain MAW843 (resistant to > 50 µg ml⁻¹ on agar plates). The low level of resistance reached in the heterologous host probably precludes the use of this *M. luteus* determinant as a selection marker in *C. glutamicum* cloning vectors. Possible reasons for the low resistance level observed in *C. glutamicum* include the inefficient expression of the determinant at the transcriptional and/or translational level [suboptimal expression signal structures (promoter, ribosome-binding site), codon usage, inefficient induction]. However, since the erythromycin-resistance gene of *M. luteus* studied here encodes a 23S rRNA N-methyltransferase, another likely explanation could be that the *C. glutamicum* 23S rRNA is a poor substrate for the enzyme. It has been demonstrated for the ermC-encoded methylase from *Staphylococcus aureus* that 23S rRNAs isolated from different genera are not equally efficient substrates for *in vitro* methylation by the purified enzyme (Denoya & Dubnau, 1987).

**Possible role of non-pathogenic micrococi in the spreading of antibiotic-resistance genes**

*M. luteus* is generally regarded as being non-pathogenic for non-immunocompromised individuals. Nevertheless, the observation of antibiotic-resistance determinants in strains of this species (Eady et al., 2000; Luna et al., 1999; this work) may be of clinical importance because these determinants may be transferred to other bacteria. The fact that the protein encoded by erm(36) was found to be most similar to erythromycin-resistance determinants found in pathogenic and opportunistic strains of *Corynebacterium* and *Propionibacterium* indicates that non-pathogenic skin micrococi may share a resistance gene pool with other high-G+C Gram-positive bacteria. However, the degree of relatedness between erm(36) and other erm ORFs (up to about 53% identity at the level of amino acid sequences) was too low to suggest a recent gene-transfer event in this particular case. Nevertheless, it is easily conceivable that plasmid-bearing strains of saprophytic skin bacteria could serve as a natural reservoir for resistance genes. Via intergeneric gene transfer such strains could contribute to the spreading of antibiotic-resistance determinants to medically important organisms. In this context it is interesting to note that naturally transformable strains of *M. luteus* have been described (Kloos, 1969; Kloos & Schultes, 1969). Thus, some *M. luteus* strains may acquire resistance factors via natural transformation.

To our knowledge, this is the first detailed characterization of an inducible antibiotic-resistance gene from the genus *Micrococcus*. It will be interesting to compare other erythromycin-resistance genes from micrococi with determinants found in other high-G+C Gram-positive bacteria from microbial communities on the skin.

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**Micrococcus luteus** macrolide resistance plasmid