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Molecular Characterization of Ampicillin-Resistant Enterococcus faecium Isolates from Hospitalized Patients in Norway

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The genetic relationship of 81 ampicillin-resistant and 21 ampicillin-susceptible Enterococcus faecium isolates from clinical infections and rectal screening in hospitalized patients in Norway was studied by pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). PFGE showed 55 different banding patterns, and 65 of the isolates could be grouped into one large group. With AFLP, 46 patterns were discerned, and 74 isolates clustered in one group. In general, the isolates had a higher degree of similarity than with PFGE. The purK gene, which is one of the targets of the E. faecium multilocus sequence typing scheme, was sequenced. Eleven different purK alleles could be discerned, with the majority of isolates (n = 80) harboring allele 1. With only two exceptions, all strains carrying purK-1 clustered in the same PFGE and AFLP groups, indicating a good correlation between PFGE type, AFLP type, and purK allele. Genetic polymorphism of a 571-bp PCR fragment of the C-terminal domain of the penicillin-binding protein 5 gene (pbp5) was determined, and sequence differences were associated with the level of ampicillin resistance. This study indicates that the majority of ampicillin-resistant E. faecium strains in Norway belong to a distinct genetic lineage of closely related genotypes. Rectal and clinical isolates were generally indistinguishable, and differences in clonal distribution and allele polymorphism were found mainly between ampicillin-resistant and -susceptible isolates.

Enterococci are common causes of nosocomial infections (20). Although they are considered a trivial cause of infection by some, the emergence of high-level ampicillin resistance, high-level aminoglycoside resistance, and glycopeptide resistance has forced us to reconsider the importance of these organisms (15). There have been several reports on the emergence of resistant enterococci in Scandinavia, but so far glycopeptide resistance is uncommon (10, 12, 27, 30, 31). Before 1995, acquired resistance in enterococci was rarely seen in Norway, but since then there has been an increase in ampicillin resistance, and more recently glycopeptide resistance has been reported within an outbreak of ampicillin-resistant enterococci (11). A report from Finland indicated that the vanA and vanB genes were incorporated into an endemic ampicillin-resistant strain in 1996 (28). Ampicillin resistance in E. faecium is associated with the production of low-affinity penicillin-binding protein 5 (PBPs). High-level ampicillin resistance may be due to either increased expression of PBPs or alterations in the pbp5 gene resulting in lower affinities for ampicillin (23, 26).

In order to characterize ampicillin-resistant E. faecium in Norway among in-patients, a point prevalence investigation was performed from March to October 1999. Due to low numbers of clinical infections with these organisms in Norway (12), it was decided to screen for rectal carriage. Eight hundred fifty-four patients hospitalized in the medical, surgical, oncological, gynecological, and pediatric departments at 10 major hospitals in Norway were screened. The epidemiology of this screening has been reported previously (13). A total of 58 ampicillin-resistant E. faecium and eight ampicillin-susceptible E. faecium isolates that were initially misclassified as resistant were found. In addition to the rectal isolates, one of the participating hospitals also supplied 36 E. faecium isolates from clinical infections that had been collected during the period from 1997 to 1999.

In the present study, the genetic relationship between ampicillin-resistant and -susceptible isolates recovered by rectal screening and from clinical infections was determined by two different genotyping schemes, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) and by sequencing of the purK housekeeping gene, which is one of the targets of the recently described E. faecium multilocus sequence typing scheme by Homan et al. (14). Furthermore, the C-terminal part of the pbp5 gene was sequenced in order to correlate sequence alterations in this region with ampicillin resistance levels. Finally, we wanted to investigate if the esp gene, a reported marker for epidemic vancomycin-resistant E. faecium strains (34), was present in our collection of glycopeptide-sensitive E. faecium strains.

MATERIALS AND METHODS

Bacterial isolates. Isolation of E. faecium from rectal samples from inpatients at 10 geographically spread major hospitals in Norway (n = 66) was performed by the use of selective medium as described previously (13). Isolation of E. faecium from clinical samples at one of the participating hospitals (Haukeland University Hospital) was done with standard laboratory methods. During the period 1997 to 1999, 21 blood cultures yielded E. faecium, and all of these were included, as were 15 randomly selected isolates from other samples (urine [n = 13] and pus [n = 2]). The isolates were identified as E. faecium by standard biochemical tests (8), and identifications were verified by means of a PCR
isolates were considered to have identical AFLP patterns when the similarity was 50 nucleotides were used for comparison. The Pearson coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis. The visual analysis revealed that isolates with over 95% similarity of the Dice coefficient were identical. The patterns comprised 13 to 17 different sized DNA fragments between 50 and 1,000 kb. Cluster analysis revealed one large group (PFGE-I) with 65 isolates that had an intragroup band variation of up to six bands. Isolates in this group differed, with seven or more bands from the isolates outside this group. No clustering according to year or site (rectal or clinical) was found. The results of the PFGE are given in Fig. 1. Amplified fragment length polymorphism. The 102 isolates comprised 46 different AFLP types with less than 95% similarity (Fig. 2). When examining isolates sharing ≥80% of the restriction fragments, a criterion used previously to discern AFLP groups (3), three groups containing three or more isolates could be discriminated. Of these, one large group contained 74 isolates. This group included the isolates grouped into the PFGE-I group except for two isolates and included 11 isolates that were not in this PFGE group.

**RESULTS**

**Bacterial isolates.** One hundred two strains from 102 patients were included. Sixty-six were from rectal samples collected from hospitalized patients in the medical, surgical, oncological, gynecological, pediatric, and intensive care unit departments at 10 major Norwegian hospitals during the point prevalence study, March to October 1999. Thirty-six were from clinical infections (blood, n = 21; urine, n = 13; and pus, n = 2) from hospitalized patients in the medical, surgical, oncological, pediatric, intensive care unit, and burn unit departments in one of the participating hospitals collected between 1997 and 1999. This hospital has had an endemic situation with ampicillin-resistant *E. faecium* since 1995.

**Pulsed-field gel electrophoresis.** The 102 isolates had 55 different banding patterns by PFGE as discerned by visual inspection. The visual analysis revealed that isolates with over 95% similarity of theDice coefficient were identical. The patterns comprised 13 to 17 differently sized DNA fragments between 50 and 1,000 kb. Cluster analysis revealed one large group (PFGE-I) with 65 isolates that had an intragroup band variation of up to six bands. Isolates in this group differed, with seven or more bands from the isolates outside this group. No clustering according to year or site (rectal or clinical) was found. The results of the PFGE are given in Fig. 1.

**Amplified fragment length polymorphism.** The 102 isolates comprised 46 different AFLP types with less than 95% similarity (Fig. 2). When examining isolates sharing ≥80% of the restriction fragments, a criterion used previously to discern AFLP groups (3), three groups containing three or more isolates could be discriminated. Of these, one large group contained 74 isolates. This group included the isolates grouped into the PFGE-I group except for two isolates and included 11 isolates that were not in this PFGE group.

**purK alleles.** Eleven *purK* alleles were found (Table 2). Allele 1 dominated and was found in 80 isolates; 26 were clinical isolates and 54 were rectal isolates. Six were ampicillin susceptible and 74 were ampicillin resistant. The ampicillin-susceptible isolates had predominantly other alleles. The vast majority of isolates that grouped in PFGE-I and AFLP-I carried the *purK-I* allele, while the other isolates mainly contained other *purK* alleles.

**Antimicrobial susceptibility.** The susceptibility of all isolates to seven antibiotics was tested, and the susceptibility data are shown in Table 3. Ciprofloxacin resistance was more common among the ampicillin-resistant isolates regardless of whether they were from clinical or rectal samples. Resistance to quinupristin/dalfopristin and high-level aminoglycoside resistance were found exclusively among ampicillin-resistant rectal isolates. No resistance to linezolid or glycopeptides was detected.

**pbp5 alleles.** Sequence heterogeneity of the C-terminal part of *pbp5* was determined for all 102 isolates. Nineteen different C-terminal alleles encoding 12 different amino acid sequences were discerned (Table 4). Twenty-five isolates harboring an aspartic acid insertion and one isolate harboring a serine insertion just after Ser-466 (GenBank accession no. X98460) were all ampicillin resistant, but the level of resistance varied from 16 to >256 mg/liter. The association between this insertion and ampicillin resistance was statistically significant, as measured by Fisher’s exact test (P = 0.0014). In addition, the
485M→T substitution in 73 of 81 resistant isolates and in 4 of 21 susceptible isolates ($P < 0.0005$), the 496N→K substitution in 78 of 81 resistant isolates and in 8 of 21 susceptible isolates ($P < 0.0005$), the 499A→T substitution in 78 of 81 resistant isolates and in 7 of 21 susceptible isolates ($P < 0.0005$), the 525E→D substitution in 78 of 81 resistant and in 8 of 21 susceptible isolates ($P < 0.0005$), the 586V→L substitution in 71 of 81 resistant isolates and in 8 of 21 susceptible isolates ($P < 0.0005$), and the 629E→V substitution in 78 of 81 resistant isolates and in 6 of 21 susceptible isolates ($P < 0.0005$) were also found significantly more often in resistant compared to susceptible strains. We considered a MIC of ampicillin of ≥16 mg/liter to indicate resistance, as recommended by the NCCLS (22).

**esp PCR and hybridization.** Three strains were repeatedly positive by esp PCR and were positive when hybridized with the esp probe. Two of the strains were closely related by PFGE and AFLP and were isolated at the same hospital on the same day. The third isolate was isolated at a different hospital and was not genetically closely related. The isolates had the purK-1 allele but different pbp5 alleles. The esp-positive strains are marked in Fig. 1 and 2.

**DISCUSSION**

In this study, the genetic relationship of ampicillin-resistant and ampicillin-susceptible *E. faecium* isolates recovered from hospitalized patients was analyzed with different molecular typing schemes. Both PFGE and AFLP clustered the majority of ampicillin-resistant isolates in a large genogroup (PFGE-I and AFLP-I), whereas the susceptible isolates seemed to be genetically more diverse. This illustrates that in this study, the grouping of isolates by both typing schemes correlates well and that the two methods have a similar discriminatory power. Earlier studies indicated that PFGE is a more discriminatory method than AFLP for studying the nosocomial spread of vancomycin-resistant enterococci (5), and PFGE has been proposed as the method of choice for epidemiological typing of vancomycin-resistant *E. faecium* by many researchers (16, 18, 19).

AFLP, first described by Vos and collaborators (32), is, however, much less labor intensive and makes it possible to test large numbers of isolates with an acceptable workload. Furthermore, AFLP typing, in contrast to PFGE, permits the study of genetic relationships among dissimilar, nonepidemiologically related vancomycin-resistant enterococci (35). This, together with our findings, suggests that AFLP is suitable for microepidemiological as well as global epidemiological studies to study the global spread of specific virulent or multiresistant strains. The isolates were not clustered according to origin (i.e., clinical versus rectal), indicating that the same bacterial populations were found in rectal carriers and in patients with

FIG. 1. PFGE dendrogram of 102 isolates produced following Dice and UPGMA analysis of Smal-digested DNA. One large group (I) could be discerned (see text). Distribution of the isolates according to hospital site (hospital codes refer to the 10 hospitals that participated), *esp* positivity, year of isolation, source of isolation, purK and pbp5 alleles, ampicillin susceptibility, and AFLP groups I to III are shown.
clinical infection. This is in line with studies that have implicated colonization as one of the important factors for dissemination of these bacteria in hospitals (2, 37).

The ampicillin-susceptible isolates were genetically more diverse than the ampicillin-resistant isolates. However, one has to be cautious when interpreting this result because the number of susceptible isolates was small and the majority of these were from clinical samples from only one of the participating hospitals. The finding, however, seems to support the notion that many of the resistant strains, even though they were sampled from different hospitals and with no apparent link, were more related than the susceptible strains, and this indicates that ampicillin-resistant strains have recently spread epidemiologically in Norwegian hospitals. A similar finding was described for vancomycin-susceptible, ampicillin-resistant enterococci (4, 14).

The allele purK-1 was the most common purK allele and was present in 80 of the 102 isolates. The fact that purK-1 was the dominant allele among isolates that were grouped together by PFGE (PFGE-I) and AFLP (AFLP-I) supports the conclusion that a clonal lineage of highly similar ampicillin-resistant *E. faecium* strains is spreading in Norwegian hospitals. Earlier findings by Homan et al. showed that vancomycin-resistant enterococcal isolates related to hospital outbreaks shared the purK-1 allele (14). The finding of the purK-1 allele among highly genetically similar isolates in the present study indicates

### TABLE 2. purK allelic polymorphism of 102 isolates of *E. faecium*

<table>
<thead>
<tr>
<th>purK allele</th>
<th>Sample</th>
<th>No. of isolates with ampicillin phenotype:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>1</td>
<td>Blood, urine, pus</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Blood</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Blood</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Blood</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3</td>
</tr>
<tr>
<td>19</td>
<td>Blood</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
</tr>
<tr>
<td>12, 15–18, 20</td>
<td>Blood</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Rectal swab</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4</td>
</tr>
</tbody>
</table>

FIG. 2. AFLP dendrogram of 102 isolates produced following Pearson and UPGMA analysis. Three AFLP groups (I to III) comprising at least three isolates were formed at ≥80% similarity. Distribution of the isolates according to hospital site (hospital codes refer to the 10 hospitals that participated), *esp* positivity, year of isolation, source of isolation, purK and *pbp5* alleles, ampicillin susceptibility, and PFGE group I are shown.
and clinical isolates revealed that more antibiotic resistance was found among the rectal isolates. This was illustrated by the fact that isolates with reduced susceptibility to quinupristin/dalfopristin and high-level resistance to gentamicin were only found among rectal isolates, while the level of ciprofloxacin resistance was high among both rectal (85%) and clinical (86%) isolates. The finding of quinupristin/dalfopristin resistance among <i>E. faecium</i> is unexpected because this drug has not been used in humans in Norway before or during the study, nor is there any veterinary use of virginiamycin in Norway that may select for quinupristin/dalfopristin resistance among <i>E. faecium</i> isolates (33). Whatever the reason for the resistance to this new drug among the ampicillin-resistant <i>E. faecium</i> strains that colonize patients in our setting, we will follow the development in clinical samples in the future. Ciprofloxacin resistance was more common among the ampicillin-resistant isolates. This is in agreement with findings by Torell et al., who concluded that over 90% of ampicillin-resistant enterococci carrier strains were resistant to fluoroquinolones (30). Ampicillin-resistant isolates (33). Whatever the reason for the resistance to this new drug among the ampicillin-resistant <i>E. faecium</i> strains that colonize patients in our setting, we will follow the development in clinical samples in the future. Ciprofloxacin resistance was more common among the ampicillin-resistant isolates. This is in agreement with findings by Torell et al., who concluded that over 90% of ampicillin-resistant enterococci carrier strains were resistant to fluoroquinolones (30). Ampicillin-resistant isolates (33). Whatever the reason for the resistance to this new drug among the ampicillin-resistant <i>E. faecium</i> strains that colonize patients in our setting, we will follow the development in clinical samples in the future. Ciprofloxacin resistance was more common among the ampicillin-resistant isolates. This is in agreement with findings by Torell et al., who concluded that over 90% of ampicillin-resistant enterococci carrier strains were resistant to fluoroquinolones (30).

### Table 3. Antimicrobial susceptibility of 102 <i>E. faecium</i> isolates

<table>
<thead>
<tr>
<th>Isolate type</th>
<th>Agent</th>
<th>MIC (mg/liter)</th>
<th>No. of isolates</th>
<th>MIC (mg/liter)</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>50% 90%</td>
<td>S I R</td>
<td>Range</td>
<td>50% 90%</td>
</tr>
<tr>
<td>Amoxicillin resistant</td>
<td>Ampicillin</td>
<td>24–256</td>
<td>64 128</td>
<td>—</td>
<td>6–&gt;256</td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
<td>0.75–&gt;32</td>
<td>&gt;32 &gt;32</td>
<td>6 5 47</td>
<td>3–&gt;32</td>
</tr>
<tr>
<td></td>
<td>Linezolid</td>
<td>0.75–2</td>
<td>1 1 58</td>
<td>0 0</td>
<td>0.5–1.5</td>
</tr>
<tr>
<td></td>
<td>Quinupristin/dalfopristin</td>
<td>0.25–64</td>
<td>0.5 1.5 50 4 4</td>
<td>0.19–0.5</td>
<td>0.5 0.5 23 0 0</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>0.5–2</td>
<td>1 2 58</td>
<td>0 0</td>
<td>0.5–2</td>
</tr>
<tr>
<td></td>
<td>Telcoplanin</td>
<td>0.094–2</td>
<td>0.25 1.5 58</td>
<td>0 0</td>
<td>0.064–1.5</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>2–&gt;1024</td>
<td>8 12 55</td>
<td>— 3</td>
<td>2–16</td>
</tr>
</tbody>
</table>
cillin-resistant enterococci carriage in that study was correlated with the use of fluoroquinolones. However, fluoroquinolone-resistant, ampicillin-resistant enterococci have also been isolated from infections in patients in Tanzania, where fluoroquinolones are less used (Bjorn Blomberg, personal communication). Thus, co-resistance to ampicillin and fluoroquinolones in *E. faecium* is an issue that merits further investigation.

Nucleotide polymorphism in the *pbp5* gene was associated with ampicillin resistance. It was striking that all the isolates with an extra amino acid in position 466 were resistant. This is most likely not a clonal phenomenon, since some of these were clonally unrelated as determined by PFGE and AFLP, but an indication that the aspartic acid and serine insertion at this position may affect the affinity of beta-lactam antibiotics for PBP5. Insertions of aspartic acid and serine at this position in strains with an increased level of resistance to ampicillin have been described previously (24, 29). Whether this reflects a causal association needs to be elucidated. Other studies have associated the presence of point mutations in the C-terminal region with certain levels of resistance (17, 24). We also found statistically significant associations between point mutations in ampicillin-resistant and -susceptible isolates in positions that earlier have been related to ampicillin resistance, such as 485M→T, 496N→K, 499A→T, 525E→D, 586V→L, and 629E→V. However, recent studies have indicated that specific point mutations alone do not entirely explain the differences in levels of resistance and that the mechanisms by which ampicillin resistance is expressed are more complex (23, 26).

To summarize, this study indicates that the majority of ampicillin-resistant enterococci in hospitals in Norway belong to a distinct clonal lineage of genetically highly similar strains. The *purK-I* allele seems to be associated with glycopeptide-sensitive *E. faecium* strains with an epidemic potential similar to what was reported earlier in glycopeptide-resistant *E. faecium* strains. Ampicillin-resistant *E. faecium* isolates in Norwegian hospitals are more resistant to other antibiotics than ampicillin-susceptible isolates. Rectal and clinical isolates are in general genetically indistinguishable, and the greatest differences in resistance traits, clonal distribution, and allele polymorphism seem to be between ampicillin-resistant and ampicillin-susceptible strains. The exact mechanisms of ampicillin resistance and the distribution and importance of *esp* in the epidemic spread of vancomycin-susceptible but ampicillin-resistant *E. faecium* requires further study.

ACKNOWLEDGMENTS

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