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Rapid Identification of *Enterococcus hirae* and *Enterococcus durans* by PCR and Detection of a Homologue of the *E. hirae mur-2* Gene in *E. durans*

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During an evaluation of PCR for identification of isolates of *Enterococcus hirae*, a homologue with 82% identity to *E. hirae mur-2* was identified in *Enterococcus durans* and was named *mur-2*$_{ed}$. PCR using primers for two genes (*copY* and *murG*) of *E. hirae* strains showed amplification with *E. hirae* strains only. PCR (under high-stringency conditions) with primers for the *mur-2*$_{ed}$ gene gave the expected amplification product only with *E. durans* strains. A combination of *murG* and *mur-2*$_{ed}$ primers in a multiplex PCR assay differentiated *E. hirae* from *E. durans* in all cases. PCR using these primers appears to be a rapid alternative for identification of *E. hirae* and *E. durans* isolates.

Phenotypic and biochemical similarities among many enterococcal species and the existence of strains with unusual or aberrant phenotypes make accurate species identification a challenge. PCR detection of the *ddl* genes (9, 10, 14), rRNA sequence-based testing (1, 6, 7, 21), direct sequencing of the *groES* gene (20), commercially available API 20 S strips (BioMerieux, SA, Plainview, N.Y.), and a nonradioactive DNA probe (AccuProbe culture identification tests; Gen-Probe, Inc., San Diego, CA) have all been used for identification of *Enterococcus* spp. (4), but these systems have focused mainly on identification of *Enterococcus faecalis* and *Enterococcus faecium*, which account for over 90% of clinical isolates belonging to this genus (16). Other species such as *Enterococcus durans* and *Enterococcus hirae* may not be recognized because many laboratories do not routinely identify enterococci to the species level. In addition, distinguishing some species such as *E. hirae* and *E. durans* poses difficulty due to phenotypically aberrant strains (21) and/or variation in the sugar fermentation profiles (5, 11, 12). Molecular methods like PCR directed to the *ddl* genes of *E. durans*/*E. hirae* (14), sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of whole-cell protein, tRNA interpacer PCR, and arbitrarily primed PCR analysis (6) have successfully identified *E. durans*/*E. hirae* isolates. With the exception of PCR, these techniques are difficult to implement in the clinical laboratory.

In the present study, we explored the utility of primers designed from some of the gene sequences of *E. hirae* available in GenBank for PCR identification of this species. During the study, we also detected a homologue of a muramidase gene (*mur-2*) of *E. hirae* (accession no. M77639) in an *E. durans* isolate and evaluated the use of primers designed from this gene (designated *mur-2*$_{ed}$) for PCR identification of *E. durans* isolates.

Bacteria used in the study were 15 isolates previously identified as *E. durans* (from Spain, the United States, and Canada) including ATCC 49479 (American Type Culture Collection [ATCC], Manassas, VA) and 5 as *E. hirae* (from Spain, the United States [including ATCC], Argentina, and Switzerland), some of which were provided by R. R. Facklam, Centers for Disease Control and Prevention, Atlanta, GA; C. Bantar, Buenos Aires, Argentina; N. Liassine, Geneva, Switzerland; and G. J. Tyrrell, Alberta, Canada. Other enterococci used in the study were 170 *E. faecalis* isolates, 181 *E. faecium* isolates, 1 *E. avium* isolate, 5 *E. casseliflavus* isolates, 5 *E. gallinarum* isolates, 5 *E. mundtii* isolates, 5 *E. raffinosus* isolates, and 2 *E. solitarius* isolates from our collection (15). The majority of these clinical isolates came from the United States, but some were from Thailand, Argentina, Colombia, Belgium, and Spain.

PCR amplification was carried out to determine the specificity of primers (Table 1) selected from the sequences of *copY*, *murG*, and *mur-2* genes of *E. hirae* (2, 8, 13, 17) available in GenBank. Following extraction of genomic DNA (250 ng) (22), the PCR conditions used included 70 pmol of the above-mentioned primers; 200 μM each of dATP, dCTP, dGTP, and dTTP; 60 mM Tris-HCl (pH 8.5); 2 mM $MgCl_2$; 15 mM ammonium sulfate; and 2 U *Taq* polymerase (Platinum *Taq* polymerase; Invitrogen). Initial (low-stringency PCR) cycling parameters were as follows: 1 cycle of a 2-min denaturation at 94°C; 35 cycles consisting of a 1-min denaturation at 94°C, a 2-min annealing at 55°C, and a 3-min extension at 72°C; and one final extension of 10 min at 72°C. The PCR protocol was also applied to fresh colonies from the agar plate containing a pure culture. The colony suspension was prepared by mixing several colonies in 100 μl of Tris-EDTA (pH 8.0), boiling 10 min, and vortexing vigorously. One microliter of the suspension was used as template for the PCR experiments.

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TABLE 1. Oligonucleotides used for PCR identification of *E. durans* and *E. hirae* isolates

Gene	PCR product (bp)	Primer name	Primer sequence	Position	Accession no.
mur-2$_{ed}$	177	*mur-2*$_{ed}$-F	+AACAGCTTACTTGACTGGACGC	126–147	AF46783
		mur-2$_{ed}$-R	−GTATTGGCGCTACTACCCGTATC	302–279	
mur-2	521	*mur-2*-F	+CGTCAGTACCCTTCTTTTGCAGAGTC	544–569	M77639
		mur-2-R	−GCATTATTACCAGTGTTAGTGGTTG	1064–1040	
copY	344	*copY*-F	+CGAGTTATCTGGACTTTAGGTCAAGC	1313–1338	Z46807
		copY-R	−CATTCAATCGTTTCGACTGGCT	1656–1635	
murG	521	*murG*-F	+GGCATATTTATCCAGCACTAG	6506–6526	Y13922
		murG-R	−CTCTGGATCAAGTCCATAAGTGG	7026–7004	

In later experiments, high-stringency PCR (HS-PCR) conditions were used for *E. hirae* identification with *mur-2* primers. The cycling conditions for the HS-PCR protocol were as follows: 1 cycle of a 2-min denaturation at 94°C; 30 cycles consisting of a 1-min denaturation at 94°C, a 15-second annealing at 60°C, and extension for 1 min 30 seconds at 72°C; and one final extension of 7 min at 72°C. For *E. durans* identification with *mur-2*$_{ed}$ primers, the HS-PCR conditions were similar to PCR with *E. hirae* except that the annealing temperature was raised to 61°C. The PCR products were analyzed by automated DNA sequencing using a model 377 DNA sequencer (ABI, Foster City, CA). Sequence analysis was done using the BLAST network service of the NCBI and the GCG software package (Genetics Computer Group, Madison, WI). The PCR product amplified from the genomic DNA of *E. durans* using *mur-2* primers by decreasing the annealing temperature to 47°C for 2 min was cloned into the pCR2.1 vector of the TA cloning kit (Invitrogen, San Diego, CA) and subsequently sequenced. The PCR product amplified from *E. durans* using *mur-2*$_{ed}$ primers and a previously cloned *mur-2* gene of *E. hirae* (2) were also used as DNA probes along with *efaA* and *aac(6′)-Ii* for *E. faecalis* and *E. faecium*, respectively (3, 19), to hybridize to DNA from 393 enterococci lysed on a nylon membrane using the conditions published previously (19). Results from PCR identification of a subset of 13 enterococci including seven *E. durans* isolates, one alleged *E. durans* isolate (ATCC 49479), one *E. durans* isolate (W185) with an aberrant phenotype (21), and four *E. hirae* isolates were also compared with results from phenotypic identification to evaluate the specificity of primers for accurate identification. Six additional previously identified *E. durans* isolates, one *E. hirae* isolate, and eight other species of enterococci were also included in low-stringency PCR and HS-PCR identification tests. For phenotypic identification of *E. hirae* and *E. durans*, previously published criteria were used (11, 12).

PCR with primers for *copY*, *murG*, and *mur-2* genes of *E. hirae* resulted in amplification of products from all *E. hirae* isolates (total DNA and colony suspension), the sequences of which showed 99% identity by GAP analysis to their respective genes in GenBank. Of the other enterococci, only *E. durans* showed any amplification product with *mur-2* primers, while use of *copY* and *murG* did not result in any amplification product. The nucleotide sequence from the *E. durans mur-2* PCR product (521 bp) showed 84% identity to nucleotides 544 to 799 of *mur-2* (accession no. M73369) of *E. hirae*. The predicted amino acid sequence of Mur-2$_{ed}$ showed 86% similarity and identity to the Mur-2 protein of *E. hirae* by GAP analysis. Based on this similarity to the *mur-2* sequence of *E. hirae*, this gene was designated *mur-2*$_{ed}$. Colony lysate hybridization with 15 *E. durans* isolates, 5 *E. hirae* isolates, 170 *E. faecalis* isolates, 181 *E. faecium* isolates, 5 *E. casseliflavus* isolates, 5 *E. raffinosus* isolates, 4 *E. gallinarum* isolates, 1 *E. avium* isolate, 5 *E. mundtii* isolates, and 2 *E. solitarius* isolates demonstrated the species specificity of the *mur-2*$_{ed}$ and *E. hirae mur-2* (2) probes, as they specifically hybridized to their respective species but not to other enterococcal species tested.

Based on the specificity of gene probes for *mur-2*$_{ed}$ and *mur-2*, we retested all the *E. durans* isolates including ATCC 49479 using HS-PCR and *mur-2*$_{ed}$ primers and identified 14/15 isolates as *E. durans*, 8 of which are shown in Table 2. Isolate

TABLE 2. Results of *E. durans* and *E. hirae* identification by phenotypic tests and/or HS-PCR

Organism(s)	Result[a] of test for[b]:								Identification by:		
	ARG	ARA	MAN	PYU	RAF	SBL	SOR	SUC	Phenotype	HS-PCR	
										mur-2$_{ed}$ primers	*mur-2*, *copY*, *murG* primers
B108, B313, ATCC 6056, SS661, SS1225, CDC2934-77, CDC2691-77	+	−	−	−	−	−	−	−	*E. durans*	*E. durans*	Non-*E. hirae*
W185	+	+*	+†	−	+*	+*	+*	+*	*E. raffinosus*	*E. durans*	Non-*E. hirae*
TX0301, TX0302, B378, SE32B	+	−	−	−	+	−	−	+	*E. hirae*	Non-*E. durans*	*E. hirae*
ATCC 49479[c]	+	−	−	−	+	−	−	+	*E. hirae*	Non-*E. durans*	*E. hirae*

[a] *, unusual reaction for the species; †, <3% of strains show these reactions. Unless otherwise noted, the results obtained with these strains were typical (10).
[b] Abbreviations: ARG, arginine; ARA, arabinose; MAN, mannitol; PYU, pyruvate; RAF, raffinose; SBL, sorbitol; SOR, sorbose; SUC, sucrose.
[c] Received as *E. durans* from ATCC.

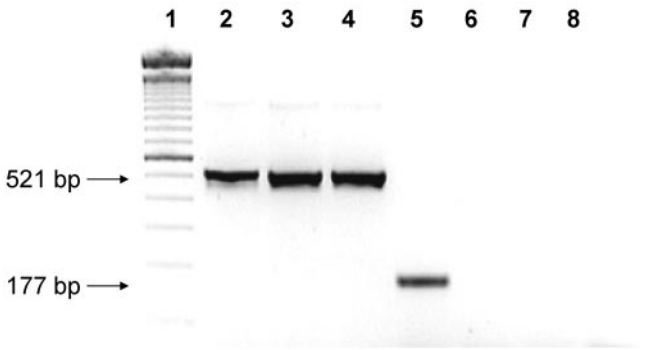

FIG. 1. Multiplex PCR of *Enterococcus* isolates using *murG* and *mur2*$_{ed}$ primers under low-stringency conditions with total DNA as the template. Lane 1, molecular weight marker; lane 2, *E. durans* ATCC 49479 (identified as *E. hirae* in this work); lanes 3 and 4, *E. hirae* clinical isolates TX2817 and TX0302, respectively; lane 5, *E. durans* ATCC 6056; lane 6, *E. gallinarum* clinical isolate; lane 7, *E. faecalis* OG1RF; lane 8, *E. faecium* TX0016.

ATCC 49479, which was sent to us as an *E. durans* isolate, was identified as *E. hirae* by PCR, as it showed amplification with *E. hirae mur-2* primers and no amplification with *mur-2*$_{ed}$ primers; gene probe hybridization and phenotypic identification results (Table 2) also identified ATCC 49479 as *E. hirae*, supporting the utility of *mur-2*$_{ed}$ primers for accurate and rapid identification. Both *mur-2*$_{ed}$ primers of *E. durans* and *mur-2* primers of *E. hirae*, by HS-PCR, specifically amplified PCR products from their respective species (Table 2) but not from other species, demonstrating their usefulness for rapid identification if used for HS-PCR as described above. Among the other 14 isolates previously identified as *E. durans*, W185 was a known phenotypically aberrant strain (21) and, based on phenotypic tests, would have been identified as *E. raffinosus*. Our phenotypic tests on W185 were the same as those described earlier (21), while the PCR and gene probe hybridization results using *mur-2*$_{ed}$ and *mur-2* primers for PCR and the respective PCR products as DNA probes identified it as *E. durans*, in agreement with its previous identification using internally transcribed spacer PCR (21).

A multiplex PCR assay (using purified total DNA as the template) under low-stringency conditions using primers (70 pmol) targeting the *murG* and *mur-2*$_{ed}$ genes was designed to determine if it could discriminate between *E. durans* and *E. hirae* isolates. The PCR yielded the corresponding amplification products of 521 bp only for *E. hirae* isolates (including ATCC 49479) and of 177 bp only for *E. durans* isolates (Fig. 1). To test the specificity of the multiplex PCR assay, it was also performed in a mixture containing equal amounts of purified total DNA (ca. 250 ng) from *E. hirae* TX2817, *E. durans* ATCC 6056, *E. faecalis* OG1RF, *E. faecium* TX2466, and an *E. gallinarum* clinical isolate. Amplification of both 521-bp and 177-bp bands was obtained with no additional bands observed, confirming the specificity of the *murG* and *mur-2*$_{ed}$ primers for *E. hirae* and *E. durans.*

In conclusion, *E. durans* and *E. hirae* have previously been considered closely related species belonging to the same species group (5, 12, 18), and the sequence similarities between these two species in the *mur-2* gene region support this relationship. Hybridization results under high-stringency conditions using gene probes for *mur-2*$_{ed}$ and *mur-2* showed their species specificity for *E. durans* and *E. hirae*, respectively. The use of *copY*, *murG*, and *mur-2* primers for *E. hirae* and *mur-2*$_{ed}$ primers for *E. durans* and HS-PCR and/or multiplex PCR (*murG* and *mur2*$_{ed}$ primers) appears to provide a rapid and accurate identification alternative to biochemical testing.

Nucleotide sequence accession number. The *mur-2*$_{ed}$ gene sequence has been entered in GenBank under accession no. AF46783.

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