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ORIGINAL ARTICLE

Uncommitted role of enterococcal surface protein, Esp, and origin of isolates on biofilm production by *Enterococcus faecalis* isolated from bovine mastitis

Mohamed Elhadidy [a,*], **Asmaa Elsayyad** [b]

[a] *Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt*
[b] *Department of Pharmacology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt*

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Purpose: This study was conducted in order to determine the occurrence of *esp* and biofilm formation among *Enterococcus faecalis* causing mastitis isolated from different bovine and environmental origins.
Materials and methods: A total of 41 *E. faecalis* isolates were obtained from clinical mastitis before antibiotic therapy, subclinical mastitis, dried manure bedding samples, and postpartum milk samples. Isolates were screened for biofilm formation using microtiter plate method using tryptic soy broth with 0.25% glucose as media. Isolates were tested for the presence of the *esp* gene, which has been reported to be essential for biofilm formation in enterococci, by means of the polymerase chain reaction.
Results: Analysis of the relationship between the presence of *esp* and the biofilm formation capacity in *E. faecalis* showed that the *esp* gene was not identified in any of the 18 biofilm-producing *E. faecalis* isolates. Moreover, two of the three non-biofilm-producing *E. faecalis* strains were *esp* positive. In addition, the biofilm assay mean values were not changed with different origins of isolation.
Conclusions: These results suggest the following: (1) lack of strict association between the presence of *esp* and biofilm formation and (2) widespread biofilm formation capacity among different sources of *E. faecalis* isolates derived from bovine mastitis.
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Introduction

Bovine mastitis remains the most costly disease in dairy production, due to decreased milk production, increased

Abbreviations: E. faecalis, *Enterococcus faecalis*; Esp, Enterococcal surface protein.

* Corresponding author. Lecturer of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Egypt.
E-mail address: mmelhadidy@gmail.com (M. Elhadidy).

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health care costs, and increased culling and death rates.[1] *Enterococcus faecalis* is a Gram-positive commensal bacterium that inhabits the oral cavity and gastrointestinal flora of humans and animals. It represents a major agent causing environmental bovine mastitis. These environmental pathogens are opportunistic invaders of the mammary gland that live and flourish in organic bedding material, which provides the nutrients needed for bacterial survival.[2]

Many persistent and recurrent infections have been attributed to the formation of biofilms, which are structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.[3] Biofilm bacteria are differentiated from their free-swimming (planktonic) counterparts, exhibiting biofilm-specific phenotypes such as enhanced tolerance to antibiotic treatment and increased levels of genetic exchange.[4] *E. faecalis* are characterized by their the innate resistance to many commonly used antimicrobial agents and their ability to become resistant to most, and in some cases to all, presently available antibiotics, either by mutation or by incorporation of foreign genetic material.[5,6] In addition, they have been proven capable of producing biofilm.[7] The enhanced antibiotic tolerance resulting from growth in a biofilm state, coupled with the intrinsic antibiotic resistance of the organism, serve as a reservoir for the dissemination of antibiotic resistance genes, thereby making treatment of biofilm-related infections a therapeutic challenge.

The implications of biofilms in infections and drug resistance by *E. faecalis* have triggered an increasing interest in the characterization of the genes involved in biofilm formation. Despite the lack of information regarding the genetic basis of biofilm formation by *E. faecalis*, some authors underlined the role of specific cell surface protein (Esp) of *E. faecalis* in biofilm formation.[7] Esp is a large surface protein with high molecular weight of unknown function whose frequency is increased among infection-derived *E. faecalis* isolates[8] and that exhibits overall sequence similarity to Bap, a biofilm-associated surface protein of *Staphylococcus aureus*, previously reported to be involved in biofilm formation.[7–9] Esp consists of a nonrepeat N-terminal domain and a central region made up of two types of tandem repeats, which are an extensive array of highly conserved repeat blocks designated as A and C repeats followed by a C-terminal membrane-spanning and anchor domain.[10]

Unlike strains from clinical human settings, there has not been a systematic study of biofilm formation by *E. faecalis* isolated from bovine and environmental origins in relation to *esp* gene. In the present work, we studied the occurrence of *esp* and biofilm formation among *E. faecalis* causing mastitis isolated from different bovine and environmental origins for better understanding of biofilm formation and its molecular basis by this organism.

Materials and methods

Bacterial strains

A total of 41 *E. faecalis* isolates were included in the study. These isolates were obtained from clinical mastitis before antibiotic therapy, subclinical mastitis (SCM), dried manure bedding samples, and postpartum milk samples collected from three different commercial dairy farms in Egypt under complete aseptic conditions. The animals were confirmed for SCM using the California Mastitis Test. All organisms presumptively identified as enterococci by colony morphology and Gram stain were identified to the species level using API STREP (bioMerieux Durham, NC, USA). All isolates were stored at −20°C in 20% glycerol–brain heart infusion broth for further testing.

Biofilm assay

The ability of the *E. faecalis* isolates to form a biofilm on an abiotic surface was quantified as previously described[7] with some modifications in optical density (OD) reading. Briefly, *E. faecalis* isolates were grown individually overnight in tryptic soy broth (TSB) with 0.25% glucose at 37°C. The culture was diluted 1:40 in TSB–0.25% glucose, and 200 μl of this cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates (Thermo Fisher Scientific, Hudson, NH, USA). After 24 hours of static incubation at 37°C, wells were gently washed three times with 200 μl phosphate-buffered saline, dried in an inverted position for 1 hour, and stained with 1% crystal violet (CV) for 15 minutes at room temperature. CV was aspirated and wells washed 3× with sterile phosphate-buffered saline. CV was extracted from adhering bacterial cells by addition of 200 μl of 80:20 (vol/vol) ethyl alcohol/acetone. The OD value of the extracted CV was measured at 570 nm (OD_{570}) using microplate ELISA reader (BioTek, Winooski, VT, USA). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Wells were examined, and the amount of biofilm formation was scored as follows: 0 = non-biofilm formation, 1+ = moderate biofilm formation, 2+ = strong biofilm formation. Non-biofilm formation with OD_{570} is <0.5, moderate biofilm formation with OD_{570} ranged from 0.5 to 2.5, and strong biofilm formation with OD_{570} is >2.5. Uninoculated wells containing TSB with glucose (200 μl) served as the negative control. Each assay was performed in triplicate and repeated three times. The data were then averaged, and standard deviation was calculated.

Polymerase chain reaction

Three different primer sets were used in polymerase chain reaction (PCR) amplifications to detect the presence of *esp* gene in DNA purified from each tested *E. faecalis* isolate and simultaneously assess the repeat number variation among these isolates. All primer sets with their sequences are summarized in Table 1. Primers esp1 and esp3 were used to amplify a 572-bp fragment within the N-terminal region of *esp*. Primers esp46 and esp47 were used for amplifications across the A repeat region of *esp*. Primers esp2 and esp5 were used for amplifications across the C repeat region of *esp* (Integrated DNA Technologies, Coralville, IA, USA). Bacterial DNA was purified using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). PCRs were performed in a final volume of 50 μl containing 0.5 μl of both forward and reverse primers (50 pmol), 2 μl dNTPs,

Table 1 PCR primers used in this study

Primer	Sequence (5′—3′)	Amplification size (bp)	Use	Source
esp1	AGGGAACGCCTTGGTATGCTAACT	572	Forward primer for amplification of *esp* across the N-terminal region	This study
esp3	TGTTCCCGCTAACTCGTGGATGAA	572	Reverse primer for amplification of *esp* across the N-terminal region	This study
esp46	TTACCAAGATGGTTCTGTAGGCAC	Multiples of 252 bp	Forward primer for amplification of *esp* across the A repeats region	10
esp47	CCAAGTATACTTAGCATCTTTTGG	Multiples of 252 bp	Reverse primer for amplification of *esp* across the A repeats region	10
Esp2	CAGATGGATCATCTGATGAAGT	Multiples of 246 bp	Forward primer for amplification of *esp* across the C repeats region	10
Esp5	GTAACGTTACTGTTACATCTGC	Multiples of 246 bp	Reverse primer for amplification of *esp* across the C repeats region	10

PCR = polymerase chain reaction.

5 μl of 10× buffer, 1 μl $MgCl_2$ (25 mM), 0.25 μl *Taq* polymerase, and 5 μg purified DNA. Distilled water was added to bring the final volume to 50 μl. The cycling conditions were as follows: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 62°C for 45 seconds, 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes. One-tenth of the amplified reaction mixture was mixed with gel-loading buffer and electrophoresed in a 1% agarose gel, and the reaction products were visualized by ethidium bromide staining. Amplified products from *esp* were digested with *Hin*dIII or *Cla*I according to the supplier's instructions (New England Biolabs, ElDakahlia, Mansoura, Egypt).

Statistical evaluation

Differences in the OD of the biofilms among strains with different genotypes/phenotypes were analyzed for significance by the Wilcoxon test for related rankable scores using Statistica 4.1 software (StatSoft, Tulsa, OK, USA).

Results and discussion

Biofilm formation by E. faecalis isolates

A total of 41 *E. faecalis* isolates were recovered from clinically mastitic cows, subclinically mastitic cows, bedding material, and postpartum milk samples with the percentage of 31.7%%, 19.51%, 34.14%, and 14.63%, respectively (Table 2). These isolates were selected in order to study their biofilm-producing ability in polystyrene microtiter plates after CV staining and were scored as discussed in the Materials and methods section. OD_{570} readings ranged from 0.1 to 2.9. Isolates with strong and moderate production were considered biofilm producers. In the tested 41 *E. faecalis* isolates, 38 (92.68%) isolates were identified as biofilm producers ($p < 0.001$). Only three strains recovered from clinically and subclinically mastitic cows were not biofilm producers (7.31%) (Table 2). These data are nearly equal to those reported by Di Rosa et al,[11] who reported that 93% of 163 *E. faecalis* isolates derived from clinical human samples were classified as biofilm producers. These results are higher than those found by other groups (with slightly different methodologies), who reported 57%[7] and 80%[12] of *E. faecalis* isolates as positive for biofilm formation, and lower than the percentage reported in one study,[13] which classified samples with ODs of >0 as positive for biofilm formation.

Biofilm formation assay mean values are not affected by origin of isolates

Biofilm formation by *E. faecalis* isolated from different origins was quantified as previously described to determine whether the origin of *E. faecalis* isolates was related to the intensity of the biofilm formation ability. Out of the 38 biofilm-producing *E. faecalis* isolates, 12 isolates were recovered from clinical mastitis, six isolates from SCM, 14 isolates from bedding material, and six isolates from

Table 2 Occurrence of biofilm formation and presence of the *esp* gene in *E. faecalis* isolates

Origin of isolates	*Enterococcus faecalis*, no. of isolates (%)	*Enterococcus faecalis*, no. of strains/total (%)	
		Biofilm +	*esp* +
Clinical mastitis	13 (31.7%)	12/13 (92.3%)	7/13 (53.84%)
Subclinical mastitis	8 (19.51%)	6/8 (75%)	5/8 (62.5%)
Bedding	14 (34.14%)	14/14 (100%)	6/14 (42.85%)
Postpartum milk samples	6 (14.63%)	6/6 (100%)	4/6 (66.66%)
Total	41	38/41 (92.68%)	22/41(53.65%) (20 biofilm producer strains and 2 biofilm non-producer strains)

postpartum milk samples (Table 2). Bacteria from the bedding environment had similar biofilm assay mean values as those isolated from mammary glands ($p < 0.001$) (Fig. 1). Biofilm formation did not appear to be a prerequisite for colonization of the bovine mammary gland. The data reported here are in agreement with previously published results[8,12,14] showing that an ability to form biofilm is extremely common among *E. faecalis* isolates, independent of their source. *Enterococcus* species are described as "environmental opportunist" in the bovine mammary gland. A characteristic of environmental opportunist pathogens is that the frequency of many virulence factors in clinical isolates is similar to the frequency in isolates in the environment.[15]

Presence of esp and biofilm

PCR amplifications of *esp* gene was carried out using three different sets of primers to amplify three different regions of *esp* gene. PCR amplification within the N-terminal region of *esp* allowed positive amplification of 572 bp in *esp* positive isolates. PCR amplifications across the A repeat and C repeat regions revealed substantial variation in band size according to the number of repeats. This difference in size corresponded to multiples of either 252 bp (A repeats) or 246 bp (C repeats). The number of A repeat units varied from two to three, while the number of C repeat units varied from three to seven. To verify that variation occurred in the number of A repeat units, the amplified product in each instance was restricted with *Hin*dIII (which cuts once within each A repeat unit), and the restriction products were analyzed on a 1% agarose gel. In all cases, only three restriction fragments of the expected sizes (173, 252, and 261 bp) were observed. Variation in C repeat units

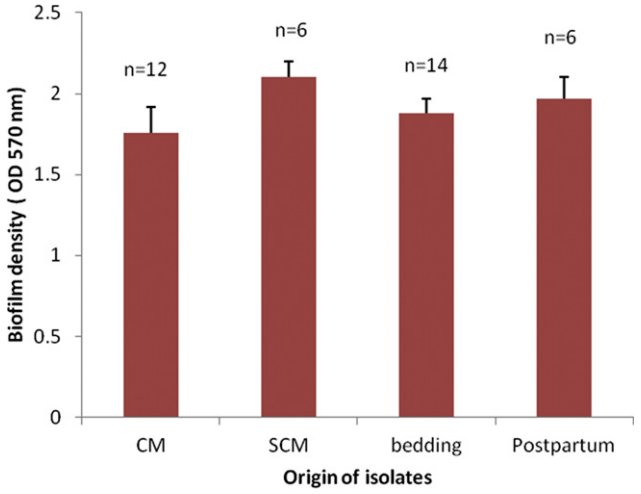

Figure 1. Biofilm formation of *E. faecalis* strains derived from different origins. The data are represented by the mean ODs of the biofilm formed by different number of biofilm producer *E. faecalis* strains isolated from different bovine and environmental sources (represented in the graph by *n*). CM = clinical mastitis; OD = optical density; SCM = subclinical mastitis. All data represent the means ± standard deviations for nine determinations (three independent experiments, each performed three times).

was similarly verified by gel electrophoresis of *Cla*I restriction fragments. In all cases, three restriction fragments of the expected sizes (196, 246, and 434 bp) were observed (data not shown).

The overall amplification of three different regions of *esp* using the three primer pairs was the same in regard to the total number of *esp* positive and negative amplifications. The *esp* gene was present in 22 of 41 *E. faecalis* isolates (53.65%). Among clinical mastitic cows, seven isolates (53.84%) were *esp* positive versus five isolates (62.5%) from subclinical mastitic cows, six isolates (42.85%) from bedding material, and four isolates (66.66%) from postpartum milk samples (Table 2). Among the biofilm producer strains (38 strains), only 20 strains were *esp* positive, and *esp* was not detected in a total of 18 biofilm-producing strains. In addition, two of the three *E. faecalis* biofilm negative strains were *esp* positive. These findings may suggest a lack of strict association between the presence of *esp* and biofilm formation. This is in contrast with other studies[7,16] reporting a significant correlation between the presence of *esp* and the capacity to form biofilms in their microtiter assay, suggesting that *esp* is involved in biofilm formation by this organism. However, in one of these reports,[7] two *esp*-deficient mutants of *E. faecalis* were found to be fully capable of biofilm formation. In addition and in the same study, it was shown that disruption of the *esp* gene completely impaired biofilm formation capacity in two *E. faecalis* strains, whereas it did not affect the biofilm formation capacity of a third strong biofilm producer *E. faecalis* strain, suggesting the existence of *esp*-dependent and *esp*-independent mechanisms for biofilm development in *E. faecalis*. On the other hand, our results are consistent with other later independent studies from different laboratories that confirmed the existence of an *esp*-independent biofilm formation pathway in *E. faecalis*.[13,17–19] Furthermore, one of these reports[18] demonstrated that *in vitro* biofilm formation occurs not only in the absence of *esp*, but also in the absence of the entire pathogenicity island that harbors the *esp* coding sequence.

In summary, our results showed that the *esp* gene does not appear to be necessary nor sufficient for the production of biofilm in *E. faecalis* isolated from bovine mastitis. We also conclude that the ability to form biofilm is extremely common among *E. faecalis* isolates, independent of their origin, and that *E. faecalis* have the phenotypic characteristics of environmental opportunists to the bovine mammary gland.

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