1 # In vivo Bioluminescence imaging for the study of intestinal

2 # colonization *by Enterococcus faecalis* in mice

3

4 ## Scientific report

5 The visit at the University College in Cork (UCC), Ireland, aimed to validate the use of the
6 Bioluminescent imaging (BLI) technology as non-invasive methods in intact animals to
7 quantify the magnitude and monitoring spatio-temporal gene expression, repetitively in the
8 same infected host for efficient functional studies of pathogenicity traits of *Enterococcus*
9 *faecalis*.
10 During the last few decades, increasing interest in *Enterococcus faecalis* has been prompted
11 by the emergence of the organism among the most frequent isolates in association with
12 hospital-related infection. Although *E. faecalis* is a natural inhabitant of the gastrointestinal
13 tract in healthy humans and some strains are used as probiotics, *E. faecalis* has been reported
14 to cause a variety of clinical syndromes associated with high mortality rates (Jett et al., 1994),
15 particularly in patients with a weakened immune system or severe underlying disease (Mundy
16 et al., 2000). The role of factors associated with the virulence of *E. faecalis* has been
17 described in several animal models (Schlievert et al, 1998; Shankar et al., 2001). While
18 reports have shown that a significant number of putative virulence traits are widespread
19 among *E. faecalis* isolates from diverse origins, gelatinase (Gutschik et al., 1979), cytolysin
20 (Coburn et al., 2003), the enterococcal surface protein Esp (Shankar et al., 2001), and
21 aggregation substance (Chow et al., 1993) are known to be enriched among nosocomial clonal
22 lineages and increase the severity of infections caused by the organism.

23 Traditional *ex vivo* methods employed to study the effect and the expression of virulence-
24 associated genes of *E. faecalis* during infection have been performed by organ extraction,

25 requiring the sacrifice of a large number of experimental animals and resulting in time-
26 consuming sample preparation for assessing cell numbers.

27 Animal and cell culture studies aimed to assess the contribution of virulence determinants in
28 disease have recently taken advantage of the development of *lux*ABCDE based
29 bioluminescence reporter systems to monitor pathogens growth and specific promoter activity
30 in vivo in a noninvasive fashion (Francis et al., 2000; Monack et al., 2004; Bron et al., 2006;
31 Riedel et al., 2007; Watson et al., 2009; Foucault et al., 2010). The main advantage of the
32 *lux*ABCDE system lies in the lack of a requirement for exogenous substrate addition. In
33 contrast to the extended half-life of the GFP reporter, the *lux*ABCDE-emitted signal is real
34 time and reflects the active transcription of selected promoters fused with the
35 bioluminescence operon. Also, bioluminescence monitoring positively contributes to the
36 implementation of two of the three "Rs" (replacement, reduction, and refinement) of ethical
37 principles in animal experimentation (Russel et al., 1959). In fact, the highly significant
38 correlation between photon emission levels and bacterial numbers allows quantifying the
39 bacterial burden within an animal infection model with high accuracy.

40 In a recent work, our group described the construction of a novel vector for conferring a
41 genetically encoded bioluminescent phenotype on different strains of *E. faecalis* (Leanti La
42 Rosa et al., 2012). This plasmid-based system provides high-level bioluminescence, does not
43 require the use of antibiotics for stable maintenance, and, although it was primarily designed
44 for *E. faecalis*, is applicable to a number of other Gram-positive species. Its functionality in *E.*
45 *faecalis* was demonstrated during growth in laboratory medium, milk, and urine and in the
46 *Galleria mellonella* infection model. This system is the first substrate addition-independent
47 reporter developed for BLI of *E. faecalis* enabling the tracking of bacterial growth and the
48 quantitative determination of specific gene expression over time during the development of
49 disease.

50 During the visit at UCC we have validated this system to investigate the spatio-temporal
51 interaction of *E. faecalis* and the murine host and the expression of cytolysin and gelatinase
52 promoter in mouse intestine and during systemic infection. The ability of the intestinal
53 microflora of conventional mice to competitively exclude experimentally introduced bacteria,
54 led us to design a model of enterococcal intestinal overgrowth based on combined use of
55 streptomycin and spectinomycin. Individually housed C57 female mice were given drinking
56 water containing 5 g/Liter of streptomycin sulfate. After 2 days of streptomycin treatment,
57 mice were returned to regular drinking water for 24 hours prior to oral gavage. Subsequently,
58 at 48h post-gavage, spectinomycin sulfate was added to the drinking water at a concentration
59 of 250 mg/L and given to the mice for the duration of the study. Bioluminescent *E. faecalis*
60 strains were grown overnight at 37°C in GM17 medium and 100 µl of washed culture were
61 administered orogastrically. Fresh fecal samples were collected at 24 hours intervals for 5
62 days, weighed, homogenized in 1 ml PBS, diluted, and plated on GM17 agar containing 500
63 mg/L spectinomycin and in Bile Esculine agar (BEA). Every day the mice were anaesthetized
64 with inhaled isoflurane and imaged using the Berthold NightOWL imaging system (Berthold,
65 Bad Wildbad, Germany). The photon emissions from bioluminescent bacteria in each animal
66 were acquired for 4 minutes using a large binning. The captured images were then quantified
67 using the indiGo™ software package (Berthold, Bad Wildbad, Germany). At 120 hours
68 postinfection, mice were sacrificed by cervical dislocation, the small and large intestines were
69 removed and the bioluminescence was detected using a Xenogen IVIS 100 system (Caliper
70 Corporation, CA). For data analysis, regions of interest (ROI) were defined and the intensity
71 of the bioluminescent signal was expressed as photons per second and quantified using Living
72 Image 3.0 software. Samples from duodenum, jejunum, ileum, cecum and colon were
73 collected, weighed, homogenized in 1 ml PBS, diluted and plated on GM17 agar containing
74 500 mg/L spectinomycin to determine the bacterial loads.

75 High levels of *E. faecalis* cells were achieved in the mice gut and they were preferentially
76 localized in the cecum and colon. This finding is consistent with previous investigations that
77 have indicated the large intestine as the principal site of colonization by enteric bacteria
78 (Poulsen et al., 1994). Moreover, low numbers of *E. faecalis* were recovered in the duodenum
79 and jejunum can be ascribable to the presence of acid, biliar and pancreatic secretions and the
80 intense peristaltic activity that prevents the bacteria to establish.
81 We examined the colonization and perpetuation abilities of the murine large and small
82 intestine of two *E. faecalis* strain isolated from different sources, namely the blood isolate
83 MMH594 and the baby fecal commensal EF62. A previous study in which the complete
84 sequence of *E. faecalis* EF62 has been analyzed revealed genomic enrichment of traits for its
85 adaptation and persistence in the intestinal environment (Brede at al., 2010). By following the
86 bioluminescence emissions intensity in fecal samples in the small and large intestine, we
87 show that *E. faecalis* EF62 has a growth advantage and persists at higher levels compared to
88 the clinical isolate; therefore, our results corroborate the previous findings. Moreover,
89 comparison of the infection abilities of *E. faecalis* TX4000 and its isogenic *phage03* deletion
90 mutant revealed no difference in the virulence potential.
91 *lux*-cytolysin and gelatinase promoters' fusion were employed to assess the expression of
92 these two virulence genes during intestinal infection. While the P*gelE* driven expression of
93 *lux*ABCDE led to no signal in the condition tested, cytolysin expression could be detected at
94 high levels providing further proof that the toxin is produced in the infection environment.
95 An intravenous mouse infection model was employed to localize the accumulation of *E.*
96 *faecalis* in intact organs by detecting the bioluminescence emission. *E. faecalis* MMH594 was
97 found in spleen, hearth and preferential accumulated in the liver and kidneys at day 4
98 postinfection. High enterococcal counts were also found in the kidneys and urine and are

99 indicative that the mice developed urinary tract infection (UTI). Consistently, cytolysin
100 expression was recorded in the organs colonized by *E. faecalis* by light emission.
101 In conclusion, we have examined and confirmed the suitability of the luciferase based reporter
102 system to monitor *E. faecalis* multiplication and virulence gene expression in intact organs
103 during gastrointestinal and systemic infection.
104 Collaboration with the host institution will continue in the future aiming to assess the
105 contribution of newly identified *E. faecalis* genes to pathogenicity during the course of
106 infection in mice through the use of BLI.
107 The results achieved at UCC will be soon included in a publication where the European
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