

Functional characterization of a gene cluster responsible for inositol catabolism associated with hospital-adapted isolates of *Enterococcus faecium*

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1 **Functional characterization of a gene cluster responsible for inositol catabolism associated with**
2 **hospital-adapted isolates of *Enterococcus faecium***

3

4 Running title: inositol metabolism in *E. faecium*

5

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18 **Keywords:** *Enterococcus faecium*, hospital-associated, inositol catabolism, ICEEfm1

19 **Repository:** Illumina MiSeq reads of two *E. faecium* strains have been deposited in the European
20 Nucleotide Archive (ENA) with accession number PRJEB43191.

21

22 **ABSTRACT**

23 *Enterococcus faecium* is a nosocomial, multidrug-resistant pathogen. Whole genome sequence
24 studies revealed that hospital-associated *E. faecium* isolates are clustered in a separate clade A1.
25 Here, we investigated the distribution, integration site and function of a putative *iol* gene cluster that
26 encodes for *myo*-inositol (MI) catabolism. This *iol* gene cluster was found as part of an ~20 kbp
27 genetic element (*iol* element), integrated in *ICEEfm1* close to its integrase gene in *E. faecium* isolate
28 E1679.

29 Among 1,644 *E. faecium* isolates, *ICEEfm1* was found in 789/1,227 (64.3%) clade A1 and 3/417 (0.7%)
30 non-clade A1 isolates. The *iol* element was present at a similar integration site in 180/792 (22.7%)
31 *ICEEfm1*-containing isolates. Examination of the phylogenetic tree revealed genetically closely
32 related isolates that differed in presence/absence of *ICEEfm1* and/or *iol* element, suggesting either
33 independent acquisition or loss of both elements.

34 *E. faecium iol* gene cluster containing isolates E1679 and E1504 were able to grow in minimal
35 medium with only *myo*-inositol as carbon source, while the *iolD*-deficient mutant in E1504
36 (*E1504ΔiolD*) lost this ability and an *iol* gene cluster negative recipient strain gained this ability after
37 acquisition of *ICEEfm1* by conjugation from donor strain E1679. Gene expression profiling revealed
38 that the *iol* gene cluster is only expressed in the absence of other carbon sources. In an intestinal
39 colonization mouse model the colonization ability of *E1504ΔiolD* mutant was not affected relative to
40 the wild-type E1504 strain.

41 In conclusion, we describe and functionally characterise a gene cluster involved in MI catabolism that
42 is associated with the *ICEEfm1* island in hospital-associated *E. faecium* isolates. We were unable to
43 show that this gene cluster provides a competitive advantage during gut colonisation in a mouse
44 model. Therefore, to what extent this gene cluster contributes to the spread and ecological
45 specialisation of *ICEEfm1*-carrying hospital-associated isolates remains to be investigated.

46

47 Abbreviations: ICE*Efm1*, integrative conjugative element *Efm1*; MI, *myo*-inositol; M1-MI, minimal

48 medium containing *myo*-inositol

49

50 INTRODUCTION

51 *Enterococcus faecium* is a commensal of the gastrointestinal tract, but also an important cause of
52 nosocomial infections, especially in immunocompromised patients (1). An important contributing
53 factor is that these *E. faecium* isolates have acquired resistance to almost all available antibiotics,
54 including ampicillin, gentamicin and vancomycin and less frequently against the more recently
55 introduced antibiotics linezolid, daptomycin and tigecycline (2). Previous whole genome sequencing
56 (WGS)-based studies revealed a split in the *E. faecium* population in a hospital-associated clade
57 (clade A) and community associated clade (clade B) (3,4). Clade A was further subdivided in clade A1,
58 mainly representing hospital-associated isolates and clade A2, mainly representing animal isolates
59 (5). Recently it became clear that the clade A2 animal isolates do not form a monophyletic subclade
60 and no longer support the split of clade A isolates into two single subclades (2,6–8). In a recent study
61 analyzing 1,644 clade A isolates, 98% of hospital associated isolates clustered in clade A1,
62 representing the most frequent source in this clade (89%) (9). Isolates clustering outside clade A1 are
63 now considered non-clade A1 (9).

64 In addition to antibiotic resistance, several virulence factors have been identified to be enriched
65 among the hospital-associated *E. faecium* isolates, including cell-wall associated proteins involved in
66 biofilm formation like the Enterococcal surface protein (Esp), a distinct subset of Secreted antigen A
67 (SagA) containing a specific number of serine-threonine repeats, the biofilm and endocarditis-
68 associated permease A (BepA) (10–12), and microbial surface components recognizing adhesive
69 matrix molecules (MSCRAMMs), see review (13). Furthermore, hospital-associated *E. faecium*
70 isolates are enriched for the presence of two genomic islands (putatively) involved in carbohydrate
71 metabolism (14,15). For one of these islands encoding for a phosphotransferase system (PTS),
72 deletion of *ptsD*, predicted to encode the enzyme IID subunit of this PTS, significantly impaired the
73 ability of *E. faecium* to colonize the murine intestinal tract during antibiotic treatment (15).

74 The aforementioned *esp* gene is encoded on *ICEEfm1* (16,17). In 2010, we published the first
75 comparative genomic study of *E. faecium*, which included in total 7 *E. faecium* genomes (18).
76 *ICEEfm1* was present in three of these genomes, including the hospital-associated isolates E1162,
77 U317 and E1679. Comparison of *ICEEfm1* in these isolates revealed the presence of a genetic
78 element of ~20 kbp in strain E1679, predicted to include a cluster of genes encoding for inositol
79 catabolism, designated as *iol* element (18). Inositol is widely found in natural environments like soil,
80 plants and aquatic environments (19) and exists in various isomeric forms such as *myo*-, *D-chiro*-,
81 *scyllo*- and *neo*-inositol. Of these, *myo*-inositol (MI) is by far the most prevalent form in nature (19).
82 In this study, we determined the presence of *ICEEfm1* and the *iol* element in a collection of 1,644
83 clade A isolates described previously (9). Furthermore, we functionally characterized the *iol* gene
84 cluster and investigated whether the capability of *E. faecium* to catabolize MI could provide those
85 strains a competitive advantage in a mouse colonization model.

86 **MATERIALS AND METHODS**

87 **Bacterial strains, plasmids and growth conditions**

88 *E. faecium* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. The *E. coli*
89 strains DH5 α (Invitrogen) and EC1000 (20) were grown in Luria-Bertani (LB) medium. *E. faecium* was
90 grown on blood agar (BA; tryptic soy agar supplemented with 5% sheep red blood cells); BD, Alphen
91 aan den Rijn, The Netherlands), in brain heart infusion (BHI) medium or supplemented with 1% *myo*-
92 inositol (BHI-MI) and in a previously described M1 medium that minimizes growth of *E. faecium*
93 when no carbon source is added and consists of 10 g of tryptone and 0.5 g of yeast extract in 1 l of
94 phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 140 mM Na₂HPO₄, 1.8 mM KH₂PO₄,
95 adjusted to pH 7.4 with HCl) (21), supplemented with different carbon sources, including 1% *myo*-
96 inositol (M1-MI) and 1% *D-chiro*-inositol (M1-DCI) at 37°C. For *E. faecium*, the antibiotics gentamicin
97 and spectinomycin were used in concentrations of 300 μ g/ml. For *E. coli*, gentamicin and

98 spectinomycin were used in concentrations of 30 µg/ml and 100 µg/ml, respectively. All antibiotics
99 were obtained from Sigma-Aldrich (Saint Louis, MO).

100 **Bioinformatic analysis**

101 In order to determine the presence of *ICEEfm1* and the *iol* element in the 1,644 clade A isolates
102 described in a previous study (9), the complete genome sequence of strain AUS0085 (accession
103 number NC_021994), which contains *ICEEfm1* including the *iol* element, was used as reference to
104 generate an ad-hoc whole genome MLST (wgMLST) scheme using Seqsphere+ version 5.0.0 (Ridom
105 GmbH, Münster, Germany, <https://www.ridom.de/seqsphere/>). In AUS0085, *ICEEfm1* encompass
106 locus tags EFAU085-02788 (encoding an LPXTG protein) to EFAU085-02871 (encoding the integrase).
107 Genome assemblies were imported into SeqSphere+ as Fasta files. Using this ad-hoc wgMLST
108 schema, the presence/absence of *ICEEfm1* and the *iol* element was determined in the set of 1,644
109 isolates and added to the metadata (Suppl. Table 1) of the 1,644 clade A isolates of the previously
110 described microreact project, thereby generating the updated project, accessible at
111 <https://microreact.org/project/pmCxZKBhMrTAL85aNKXbAh>. Patristic distances were extracted from
112 the core-genome based tree using the cophenetic function available in the R package ape (version
113 5.4-1) (22,23).

114 Next, we determined the integration site of the *iol* element in *ICEEfm1* as identified in strain *E.*
115 *faecium* E1679 (18) using the recently described Panaroo pipeline (version 1.2.3) (24). Panaroo was
116 run on 'strict' mode and the function 'panaroo-gene-neighbourhood'
117 (https://gtonkinhill.github.io/panaroo/#/post/gene_neighbourhood) was considered to explore the
118 genome graph neighborhood for the first and last gene of the *iol* element, respectively. The genome
119 sequence of *E. faecium* strain E7356 was used as reference since the entire *ICEEfm1* including the *iol*
120 element was located on a single contig. In this strain the first and last gene of the *iol* element is
121 represented by 'E7356_00286' and 'E7356_00303', respectively. Presence of putative transcription
122 terminators were predicted using RNAfold (<http://rna.tbi.univie.ac.at/>).

123 **Determination of growth curves**

124 A BioScreen C instrument (Oy Growth Curves AB, Helsinki, Finland) was used to monitor growth of *iol*
125 gene cluster-containing strains, the effects of *iolD* deletion on bacterial growth and acquisition of the
126 *iol* gene cluster in different media as indicated. All strains were grown overnight in BHI. Cells were
127 inoculated at an initial OD₆₆₀ of 0.0025 into 300 µl M1-MI and/or M1-DCI incubated in the Bioscreen
128 C system at 37°C with continuous shaking and absorbance of 600 nm (A600) was recorded every 15
129 min for 18 hours. Each experiment was performed in triplicate.

130 **Mobilization of ICEEfm1 using myo-inositol as selection marker**

131 The mobilization of ICEEfm1 was studied by performing filter-mating experiments as previously
132 described (18). The ICEEfm1-containing strain *E. faecium* E1679 was used as donor and a rifampicin-
133 and fusidic acid-resistant derivative of *E. faecium* O12 (25) designated *E. faecium* E4658 and a
134 previously used strain *E. faecium* BM4105RF (18) were used as recipient. After filter-mating, aliquots
135 of 200 µl were spread on M1-MI plates containing rifampicin and fusidic acid, both at 25 µg/ml, to
136 select for transconjugants. For quantification of the number of cells of the donor and recipient, 10 µl
137 of a 10-fold dilution series was plated on respectively M1-MI plates and BHI plates containing
138 rifampicin and fusidic acid. Transconjugation efficiency was calculated as the viable counts on the
139 M1-MI plates with rifampicin and fusidic acid divided by the viable counts on BHI plates with
140 rifampicin and fusidic acid. PFGE on SmaI-digested total DNA was performed as described previously
141 (26) to confirm that the transconjugant had the same genetic background as E4658. Lambda Ladder
142 PFGE Marker (New England Biolabs, Ipswich MA) was used to estimate the sizes of the PFGE
143 fragments. Southern blotting and probe hybridization was performed as described previously (27).
144 The probes used in the hybridizations were generated by PCR with the primer-pairs *iolA*-F and *adh*-
145 *RT-R* (Suppl. Table 2). In addition, WGS was performed to determine the integration site of ICEEfm1
146 of the recipient strain E4658 and its transconjugant E7855 as previously described (28). In brief,
147 Genomic DNA was isolated from overnight cultures in BHI broth using the Wizard Genomic DNA
148 Purification Kit (Promega), according to the manufacturer's instructions. Library preparation for

149 sequencing was performed using the Nextera XT Kit. Sequencing runs were generated using 250
150 nucleotide paired-end sequencing on the MiSeq platform (Illumina). Raw reads were trimmed,
151 assembled into contigs using SPades (vs. 3.6.2) and annotated using PROKKA (vs. 1.11 (29)). Illumina
152 MiSeq reads of these two *E. faecium* strains have been deposited in the European Nucleotide Archive
153 (ENA) with accession number PRJEB43191.

154 **Generation of a *iolD* markerless deletion mutant**

155 The previously described Cre-*lox* system was used to generate a markerless deletion mutant in the
156 *iolD* gene (30). We were unable to generate this mutant in strain E1679 in which the *iol* gene cluster
157 was first identified, since this strain was resistant to both gentamicin and spectinomycin, which are
158 selection markers for the generation of targeted mutants. As these antibiotics are required in the
159 generation of deletion mutants, we screened for other *iol* gene cluster-containing strains and found
160 that strain *E. faecium* E1504 was susceptible for both antibiotics and was therefore used to generate
161 an *iolD* mutant. For the amplification of the 5`-flanking region, we used primers *iolD*Up-F-XhoI and
162 *iolD*Up-R-EcoRI and for the 3`-flanking regions primers *iolD*Dn-F-EcoRI and *iolD*Dn-R-SmaI (Suppl.
163 Table 2). Generation of a marked deletion mutant was performed as described (30) and was
164 confirmed by PCR using the *iolD* check-up and check-down primers (Suppl. Table 2). Removal of the
165 gentamicin resistance marker was obtained by electroporation of pWS3-Cre into the marked deletion
166 mutants as described (30). Loss of the marker was confirmed by PCR using the *iolD* check-up and
167 check-down primers.

168 **RNA isolation, reverse transcription and quantitative real-time RT-PCR (qRT-PCR)**

169 In order to investigate whether the *iol* gene cluster is organized as an operon, *E. faecium* E1504 was
170 grown in M1-MI to an OD₆₀₀ of 0.3 prior to RNA isolation. To investigate growth condition-dependent
171 expression of the *iol* gene cluster, E1504 was grown in BHI, BHI-MI, and M1-MI. RNA isolation, cDNA
172 synthesis and quantitative real time PCR (qRT-PCR) was performed as previously described (10). In
173 brief, RNA was isolated using TRI Reagent (Ambion) according to the manufacturer's protocol. For

174 the operon structure, first strand synthesis using Maxima reverse transcriptase (Thermo Scientific, St.
175 Leon-Rot, Germany) was performed using gene specific primers on the 5'-end of each gene, indicated
176 with RT-R (Suppl. Table 2). The presence of intergenic cDNA was subsequently determined by PCR
177 using the same gene specific primer in combination with a 3'-end located primer of its upstream
178 located gene (Suppl. Table 2). As negative control, the same procedure for cDNA synthesis was
179 followed but without adding reverse transcriptase. As positive control for the PCR, purified genomic
180 E1504 DNA was included.

181 To determine growth condition-dependent expression of *lacl*, *iolD*, *iolA* and the *sss*-like gene, cDNA
182 was synthesized from RNA using Maxima First strand cDNA synthesis kit for RT-qPCR (Thermo
183 Scientific, St. Leon-Rot, Germany). In addition, quantitative PCRs using primers indicated with "q"
184 (Suppl. Table 2) on the synthesized total cDNAs were performed using Maxima® SYBR Green/ROX
185 qPCR Master Mix (Thermo Scientific) using a StepOne™ Realtime PCR system (Applied Biosystems,
186 Nieuwekerk a/d IJssel, The Netherlands) with the following program: 95°C for 10 min, and
187 subsequently 40 cycles of 95°C for 15 sec, 55°C for 1 min. The expression of the *tufA* gene was used
188 as a reference for the determination of relative expression levels (Suppl. Table 2)(31). This
189 experiment was performed with three biological replicates in a single technical experiment.

190 **Promoter mapping using 5' RACE**

191 *E. faecium* E1504 was grown in M1-MI to an OD₆₀₀ of 0.3. Total RNA was isolated as previously
192 described. We used the 5' RACE kit (Rapid amplification of cDNA ends, Invitrogen, The Netherlands)
193 to map the promoter of the inositol gene cluster according to the manufacturers' protocol. After first
194 strand synthesis using gene specific primers 1 (GSP1) (Suppl. Table 2), a nested PCR with GSP2
195 primers was performed to amplify the product and cloned in pGEM-T Easy TA cloning vector
196 (Promega, Madison, WI). Inserts were sequenced to determine the cDNA end.

197 ***In vivo* mouse colonization model**

198 Specific pathogen-free 10-wk-old female wildtype BALB/c mice were purchased from Charles River

199 (Maastricht, The Netherlands). The animals were housed in rooms with a controlled temperature and
200 a 12-h light-dark cycle. They were acclimatized for 1 week prior to the experiment and received
201 standard rodent chow (www.sdsdiets.com) and water ad libitum.

202 Intestinal colonization by wild-type E1504 and E1504 Δ *iolD* (inoculum of 1×10^4 CFU/300 μ l Todd
203 Hewitt Broth) was tested as previously described (10). In brief, two days before inoculation of
204 bacteria, mice were administered subcutaneous injections of ceftriaxone (Roche, Woerden, The
205 Netherlands; 100 μ l per injection, 12 mg/ml) two times daily and one time at the day of inoculation.
206 For the remaining duration of the experiment, cefoxitin (0.125 g/l) was added to sterile drinking
207 water. Collection of samples and determination of bacterial outgrowth was performed as previously
208 described (10). For statistical analysis the unpaired two-tailed Student's t-test was applied.

209 **Ethics Statement**

210 This study was approved by the Animal Ethics Committee Utrecht and the Animal Welfare Body
211 Utrecht as part of a project, which was licensed by the Central Authority for Scientific Procedures on
212 Animals (CCD) (license number: AVD115002016568).

213 **RESULTS**

214 **Identification and distribution of ICEEfm1 and the *iol* element in 1,644 clade A isolates**

215 ICEEfm1 was identified in 789/1,227 (64.3%) clade A1 isolates, including 786 hospital-associated, one
216 human non-hospital associated and two pet isolates (Fig. 1A), but in only 3/417 (0.7%) non-clade A1
217 isolates, all of which originated from hospitalized patients (Fig. 1A). The *iol* element, encoding 5
218 genes with unknown function and the *iol* gene cluster (Suppl. Fig. 1), was identified in 180/792
219 (22.7%) of the ICEEfm1-containing isolates (Fig. 1A). The integration site of the *iol* element in ICEEfm1
220 was determined by a genome graph neighborhood analysis considering the first and last genes of this
221 element (E7356_00303, E7356_00286), respectively (Suppl. Fig. 2 A and B). For the first gene
222 (E7356_00303 in the reference), in 168/180 of the *iol* element carrying isolates, the same
223 neighboring genes were identified up- and downstream (Suppl. Fig 2A). Although in 57 of these

224 isolates some variation was observed in either: i) presence of additional hypothetical genes not
225 present in E7356 and/or ii) absence of E7356 genes (e.g. E7359_00299). In 8/180 isolates, the first
226 gene of the *iol* element was found on a small contig and therefore only a small number of
227 neighboring genes could be identified (Suppl. Fig 2A). For 4/180 isolates, also due to very fragmented
228 genome assemblies, the analysis to identify the neighboring genes for the first gene of the *iol*
229 element failed. However, for all 180 isolates the gene neighboring analysis revealed the same genes
230 up- and downstream of the last gene of the *iol* element (E7356_00286) (Suppl. Fig 2B). Based on
231 these results, we assume that in all strains the *iol* element is integrated at the same site.

232 The previously generated phylogenetic tree based on the core gene alignment for 1,644 *E. faecium*
233 isolates (9) was used to determine the distribution of *ICEEfm1* with and without the *iol* element
234 among previously defined sequence clusters (SC)(9) (Fig. 1A and Suppl. Table 1). *ICEEfm1* was
235 identified among 18 SCs and most prevalent in SC1 (291/792, 36.7%) and SC13 (124/792, 15.7%) (Fig.
236 1A). The *iol* element was variably present in 13 of these 18 SCs, e.g. in *ICEEfm1* containing SC1
237 isolates the *iol* element was detected in 82/291 (28.1%) isolates, while it was absent in *ICEEfm1*
238 containing SC13 isolates (Fig. 1A). Examination of the phylogenetic tree using microreact revealed
239 several pairs of genetically closely related isolates with and without *ICEEfm1*, e.g. *E. faecium* E1469
240 (2000, Madrid, Spain) and E1738 (2002, Madrid, Spain) (*patristic distance* = $1.6e-5$) (Fig. 1B) and
241 genetically closely related *ICEEfm1* positive isolates with and without the *iol* element, e.g. *E. faecium*
242 E2359 (2004, Singapore) and E6958 (2010, Portugal) (*patristic distance* = $1.11e-4$) (Fig. 1C). These
243 findings suggest acquisition or loss of *ICEEfm1* with and without the *iol* element among hospital-
244 associated *E. faecium* isolates.

245 ***In silico* analysis of the inositol catabolic pathway as compared to *Bacillus subtilis***

246 In order to infer the potential role of the *E. faecium iol* gene cluster, we compared the organization
247 and presence/absence of *iol* genes with the previously functionally characterized *iol* gene cluster of
248 *B. subtilis*, which encodes a functional catabolic pathway for MI and DCI (32) (Fig. 2 and Suppl. Fig. 3).

249 This comparison revealed a difference in gene synteny and the absence of four genes in the *E.*
250 *faecium iol* gene cluster encoding for lolF (minor MI transporter), lolI (2-keto-myo-inositol (2KMI)
251 isomerase), lolJ (6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase) and lolH (protein with
252 unknown function) (Fig. 2). Based on the MI and DCI catabolic pathway as determined for *B. subtilis*,
253 the absence of these genes suggested that the *E. faecium iol* gene cluster might not be functional
254 (Supp. Fig. 3). However, the *E. faecium iol* gene cluster putatively encodes MI and/or DCI transporters
255 represented by the last two genes annotated as sodium/*myo*-inositol cotransporter (SSS) and sugar
256 phosphotransferase system (PTS), respectively, potentially acting as alternative for lolF or lolT (the
257 major transporter of MI in *B. subtilis* encoded outside the *B. subtilis iol* gene cluster) (Fig. 2 and
258 Suppl. Fig. 3). BLASTp search of *B. subtilis* lolT and lolJ against the complete genome of *E. faecium*
259 AUS0085 revealed the presence of (putative) alternative proteins with shared conserved domains
260 with locus-tags EFAU085_02406 (37% identity to lolT) and EFAU085_00643 (45% identity to lolJ),
261 respectively. In contrast, BLASTp revealed no proteins with significant identity or shared domains for
262 lolI.

263 In *B. subtilis*, expression of the *iol* gene cluster is regulated by a transcriptional repressor lolR
264 encoded upstream of the *iol* gene cluster (Fig. 2) (33). In *E. faecium* AUS0085, a putative
265 transcriptional regulator, annotated as Lacl, is encoded directly upstream the *iol* gene cluster (Fig. 2),
266 but with no similarity with lolR of *B. subtilis*. To determine putative domains of Lacl, a protein BLAST
267 was performed, which revealed that Lacl contains an N-terminal helix-turn-helix motive with
268 similarity to the Lacl family of transcriptional repressors (Suppl. Fig. 4). In addition, the protein
269 contains a large domain with similarity to a periplasmic sugar-binding domain, predicted to be
270 involved in the transport of sugar-containing molecules across cellular membrane. These domains
271 suggest that Lacl is the regulator of the *iol* gene cluster, but could also be involved in transport of MI
272 and/or other sugars (Suppl. Fig. 4).

273 Finally, the *E. faecium iol* gene cluster contains one gene which is absent in the *B. subtilis* gene

274 cluster, i.e. *adh*. BLAST-P analysis revealed that Adh contains domains that belong to the family of
275 iron-containing alcohol dehydrogenase, most of which have not been characterized.

276 **Growth of *E. faecium* on *myo*-inositol and *D-chiro*-inositol**

277 In order to investigate whether the *iol* gene cluster encodes for a functional MI and/or DCI catabolic
278 pathway, we generated growth curves of *E. faecium* E1679 (Fig. 3A) and another ICEEfm1- and *iol*
279 gene cluster-containing strain E1504 in minimal medium M1 containing 1% MI (M1-MI) (Fig. 3B). In
280 addition, E1504 was also grown in M1 with 1% DCI (M1-DCI) (Fig. 3B). Both strains were able to grow
281 in M1-MI, indicating that they are able to use *myo*-inositol for their metabolism, although E1679
282 seemed to grow better as this strain reached a higher final OD. In contrast, no growth was observed
283 in the presence of DCI, which is likely due to the absence of *loll* in the *E. faecium iol* gene cluster. As a
284 control, E1504 was grown in M1 in the absence of a carbon source, but as expected no growth was
285 observed comparable to growth in the presence of DCI.

286 **Generation of an ICEEfm1 transconjugant and a markerless *iolD* mutant**

287 Two different approaches were used to further confirm that the *iol* gene cluster encodes for MI
288 catabolism. For the first approach, we investigated whether the ability of MI catabolism encoded by
289 ICEEfm1 is transferable. To this aim, we used MI for the selection of transconjugants using *E. faecium*
290 E1679 as donor and *E. faecium* E4658 and the previously used *E. faecium* BM4105RF as recipient
291 strains (Table 1). No transconjugants were obtained using the BM4105RF as recipient. In contrast,
292 when using E4658 as recipient transconjugants were obtained at a frequency of 1×10^{-8} per recipient
293 cell. The transfer of ICEEfm1 to E4658 was confirmed by PFGE analysis (Fig. 4A) with subsequent
294 Southern blotting using an internal fragment of the *iol* gene cluster as probe (Fig. 4B). This revealed
295 that a single fragment in the transconjugant, which we designated E7855, had increased in size and
296 hybridized with the internal fragment of the *iol* gene cluster as probe. WGS of E4658 and E7855
297 confirmed integration of ICEEfm1 in the 3'-end of the E4658 *rpsI* gene. For the phenotypic
298 characterization, we compared the growth capabilities of the donor, recipient and transconjugant in

299 M1-MI medium (Fig. 3A). No growth was observed for the recipient E4658. However, the
300 transconjugant E7855 was able to grow in M1-MI medium similar to the donor strain E1679,
301 indicating that acquisition of the *iol* gene cluster resulted in the capability to use MI as carbon source
302 for growth (Fig. 3A).

303 For the second approach, we constructed a markerless *ioID* mutant strain in *E. faecium* strain E1504,
304 designated E1504 Δ *ioID* (Table 1). For the characterization of the E1504 Δ *ioID* mutant, we compared
305 growth capabilities of E1504 wildtype and E1504 Δ *ioID* mutant in M1-MI medium, which revealed
306 that the E1504 Δ *ioID* mutant strain was not able grow (Fig. 3B).

307 From these two approaches we can conclude that acquisition of the *iol* gene cluster results in the
308 ability to utilize MI, while deletion of *ioID* results in loss of this ability.

309 **The *iol* gene cluster is only expressed in the absence of other carbon sources**

310 Next, we determined growth medium dependent RNA expression levels on a selection of genes
311 encoded on the *iol* gene cluster, including its putative transcriptional regulator *lacl* and three genes
312 dispersed over the *iol* gene cluster, i.e. *ioID*, *ioIA* and *sss* (Fig. 2). First, the expression of the genes
313 was determined in *E. faecium* E1504 grown in BHI used as control and in BHI and M1 supplemented
314 with 1% MI, BHI-MI and M1-MI, respectively (Fig. 5). For none of the four genes, difference in
315 expression levels were observed when E1504 was grown in BHI-MI compared to the BHI control.
316 However, compared to BHI, *ioID*, *ioIA* and *sss* were highly expressed in M1-MI, but no difference was
317 observed for *lacl* (Fig. 5). These findings suggest that the *iol* genes, but not *lacl*, are only expressed in
318 the absence of other carbon sources.

319 **Transcriptional organization of the *iol* gene cluster**

320 The transcription start site of the *iol* gene cluster was identified at 32-bp upstream the *ioIC*
321 startcodon using 5'- RACE analysis (Fig. 6). A putative promoter region, including -35 and -10 boxes
322 were identified 44-bp and 69-bp upstream of the *ioIC* start codon (Fig. 6). To investigate the
323 transcriptional organization of the *iol* gene cluster, we determined whether the *iol* genes are

324 transcribed as a single RNA molecule. In total, 12 PCRs were performed on synthesized cDNA from
325 strain *E. faecium* E1504 (Suppl. Fig. 5A). The expected sizes for PCR products (indicated in Suppl.
326 Table 2) were obtained between all genes encoded on the *iol* gene cluster, except for PCR-1
327 amplifying the intergenic region of *lacl* and *iolC* and PCR-12 amplifying the intergenic region between
328 the *pts* gene and its downstream region (Suppl. Fig. 5B). A predicted transcriptional terminator is
329 located downstream the *lacl* and *pts* genes with a ΔG of -53.20 kcal/mol and ΔG of -121.40 kcal/mol,
330 respectively (Suppl. Fig. 5A, C and D). Downstream *lacl*, we identified two pairs of inverted repeats,
331 which are part of the predicted transcriptional terminator, but the inverted repeat identified
332 upstream from the -35 box is the predicted DNA binding site for the LacI repressor (Fig. 6 and Suppl.
333 Fig. 5C). Taken together, these findings indicate that *lacl* and the *iol* gene cluster are not part of the
334 same operon, but that the *iol* genes, *sss* and *pts* genes are indeed organized as a single operon.

335

336 **The *iol* gene cluster has no role in intestinal colonization in a mouse**

337 In order to investigate whether *iol* gene cluster containing strains would have a selective advantage
338 in their ability to colonize the gut, we orally inoculated two different groups of mice with either wild-
339 type E1504 or its E1504 Δ *iolD* mutant strain to compare intestinal colonization rates. Compared to
340 previous studies (10,15), we used a lower inoculum, i.e. 1×10^4 cfu in 300 μ l instead of 1×10^8 cfu in
341 300 μ l. We hypothesized that using a lower inoculum might result in clearer differences in
342 colonization rates between the two groups very early after inoculation. However, the data showed
343 that already at day 1 after inoculation similar amounts of 1×10^{10} *E. faecium* cfu/gr feces were
344 identified in both mice groups inoculated with either the E1504 wildtype or the E1504 Δ *iolD* mutant
345 (Fig. 7A). These high colonization rates remained until the end of the experiment at day 10, when
346 mice were sacrificed. Similarly, in the colon and cecum, no difference in colonization levels was
347 observed between wild-type and mutant strains. A small difference was observed in the ileum,
348 where a small reduction in colonization of the wild-type strain was observed, but this difference was
349 not statistically significant (Fig. 7B).

350 DISCUSSION

351 In this work, we describe the distribution and functional characterization of the *E. faecium iol* gene
352 cluster, which was identified as part of an ~20 kbp genetic element, designated *iol* element,
353 integrated in *ICEEfm1* of *E. faecium* E1679 (18).

354 The *iol* element was identified among 23% of the *ICEEfm1*-containing *E. faecium* isolates and these
355 *ICEEfm1*-containing *E. faecium* isolates with or without the *iol* element belonged to multiple
356 sequence clusters within clade A1. Furthermore, the *iol* element was always found integrated at the
357 same site in *ICEEfm1* as in *E. faecium* E1679. This suggests that *ICEEfm1* containing the *iol* element
358 was acquired in the early stage of the clade A1 evolution and subsequently lost in some branches
359 during evolution. Examples for such events were found by close examination of branches containing
360 pairs of genetically closely related isolates not only with and without *ICEEfm1*, but also pairs of
361 *ICEEfm1*-containing isolates with and without the *iol* element. However, we cannot exclude that
362 during the evolution of clade A1 both elements were acquired independently. Gene neighborhood
363 analysis revealed variation in gene content in the region between the integrase (first gene of
364 *ICEEfm1*) and the first gene of the *iol* element, indicating that these elements are not 100% identical
365 between isolates. In summary, it remains unclear whether both elements were acquired or lost, but
366 these findings are in line with previous findings that the genomes of *E. faecium* are highly dynamic
367 (34).

368 For the functional analysis of the *iol* gene cluster, we first performed an *in silico* comparison with the
369 *iol* gene cluster of *B. subtilis*, which has been studied in detail (32,33). This comparison revealed a
370 different gene synteny and the absence of several genes in the *E. faecium iol* gene cluster. Variation
371 in the organization of *iol* gene clusters has also been described for other gram-positive bacteria, e.g.
372 *Enterococcus faecalis* OG1RF (35), *Lactobacillus casei* BL23 (36) and *Corynebacterium glutamicum*
373 (37) and in gram-negative bacteria, e.g. *Salmonella enterica* serovar Typhimurium, *Klebsiella*
374 *pneumonia* and *Yersinia enterocolitica* (38). We further showed that despite the lack of specific *iol*
375 genes relative to *B. subtilis*, both *E. faecium* E1679 and E1504 were able to grow in minimal medium

376 with MI as sole carbon source and that the *iol* gene cluster is responsible for this phenotype. The
377 observation that the tested *E. faecium* strains were not able to grow in minimal medium with DCI can
378 be explained by the absence of the *iolI* gene. The only species that contained an identical gene order
379 compared to *E. faecium* was *E. faecalis* (35). Bourgogne *et al.* did not perform a detailed
380 characterization of the *E. faecalis iol* gene cluster, but only mentioned that transposon insertion
381 mutants in the *iolB* and *iolG2* genes failed to grow on MI (35). Their annotation of the last gene of the
382 *E. faecalis* gene cluster as *IolT*, which has 92% amino acid identity with the sodium/*myo*-inositol
383 cotransporter (SSS) protein of *E. faecium*, is likely based on the domain predictions from this protein,
384 as there is only low overall similarity with the major MI transporter *IolT* (15%) and minor MI
385 transporter *IolF* (18%) of *B. subtilis*. In addition, in *E. faecalis* a similar *lacI* gene encoded upstream
386 the *iol* gene cluster was considered as putative regulator (35). In contrast to *E. faecium*, the *E.*
387 *faecalis iol* gene cluster is not encoded on a large mobile genetic element like *ICEEfm1* but instead is
388 located on a hot spot for integration, as other types of insertion elements were identified at the
389 same position in other *E. faecalis* isolates (35,39).

390 The *iol* gene cluster in *E. faecium* is organized as an operon and transcribed as a polycistronic mRNA
391 molecule, comparable to *B. subtilis* (33). Upstream of *iolC*, we identified the transcriptional start site
392 and the putative binding sites for the assumed transcriptional repressor *LacI*. However, we were not
393 able to confirm this assumption as the generation of a *lacI* mutant strain was unsuccessful and no
394 differential expression was observed in M1-MI compared to BHI. The latter could also be the result of
395 constitutively expression of *lacI*.

396 Hospital associated *E. faecium* isolates belonging to clade A1 are able to colonize the dysbiotic gut of
397 hospitalized patients at high densities, which contributes significantly to subsequent clinical
398 infections and hospital transmission (40). In order to investigate whether the *iol* gene cluster
399 contributes to high density gut colonization, we compared the colonization capacity of *E. faecium*
400 strain E1504 and its E1504 Δ *iolD* mutant in a mouse intestinal colonization model, mimicking
401 colonization of dysbiotic gut. In this model, we did not observe a difference in the capacity to

402 colonize the gut between wild-type and the *iolD* mutant strain. Only for the small intestines, a small,
403 but not significant decreased colonization was observed for E1504 wild-type strain. Absence of MI in
404 the gut mice as an explanation for this lack of difference in colonization rate between wild-type and
405 *iol* mutant is not likely as inositol is present in the food pellets for mice according to the product
406 sheet of the food supplier (www.sdsdiets.com). Furthermore, increased inositol metabolites have
407 been found in mice treated with clindamycin (41). In our mouse colonization study, mice were
408 treated with cephalosporins, therefore we cannot directly translate the published findings to our
409 study. The most probable explanation for the lack of difference in colonization rates between wild-
410 type and *iolD* mutant is that the *iol* gene cluster is only expressed in the absence of other carbon
411 sources. In our colonization model, mice were fed with rodent chow that contained other carbon
412 sources, hence it is likely that under these conditions the *iol* gene cluster is not expressed. Regulation
413 of expression of the *iol* gene cluster by other carbon sources has also been observed for other
414 species, e.g. *S. enterica* serovar Typhimurium, *C. perfringens* and *L. casei* (36,38,42).
415 For *Legionella pneumonia* it has been shown that utilization of inositol provides this species a
416 selective advantage for intracellular survival in amoebae and macrophages (43). When inositol was
417 added to *L. pneumophila*-infected amoebae or macrophages, intracellular growth of a parental strain
418 was promoted, but not of the *iolT* or *iolG* mutant. Growth stimulation by inositol was restored by
419 complementation of the mutant strains (43). Macrophage survival has also been experimentally
420 shown for *E. faecium* (31,44). Future research should reveal whether *E. faecium* isolates containing
421 the *iol* gene cluster have a selective advantage and can persist longer in macrophages.

422

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433

434 **Conflicts of interest**

435 The authors declare no conflicts of interest.

436

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442

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- 570
- 571

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference or source
Strains		
<i>E. faecium</i>		
E1679	Clinical blood isolate; Brazil; AmpR, VanR, GenR, SpcS; ICEEfm1+	18
E1504	Clinical blood isolate; Spain; AmpR, VanS, GenS, SpcS; ICEEfm1+	9
E1504Δ <i>iolD</i>	Markerless deletion mutant of <i>iolD</i> of E1504; GenS; ICEEfm1+	This study
E4658	Pig isolate; Netherlands; RifR, FusR, VanR; ICEEfm1-	25
E7855	Transconjugant; E1679 ICEEfm1 donor, O12 acceptor	This study
BM4105RF	Recipient strain; France; RifR, FusR; ICEEfm1-	18
<i>E. coli</i>		
DH5α	<i>E. coli</i> host strain for routine cloning	Invitrogen
EC1000	MC1000 <i>glgB::repA</i>	20
Plasmids		
pWS3	Shuttle plasmid; ts in gram-positive hosts; SpcR	30
pEF39	pWS3: <i>ebrB</i> fusion with gentamicin resistance cassette cloned in the EcoRI site of the <i>ebrB</i> gene fusion fragment plasmid for generating an <i>ebrB</i> marked mutation; SpcR, GenR	This study
pEF40	pWS3: <i>esp</i> fusion with gentamicin resistance cassette cloned in the EcoRI site of the <i>esp</i> gene fusion fragment plasmid for generating an <i>esp</i> marked mutation; SpcR, GenR	This study
pWS3-Cre	pWS3 derivative expressing Cre in <i>E. faecium</i>	30

^aAmp, ampicillin; Van, vancomycin; Chl, chloramphenicol; Gen, gentamicin; Spc, spectinomycin; ICEEfm1+, *E. faecium ebrB* containing pathogenicity island

572

573

574 **LEGENDS TO FIGURES**

575 **Fig. 1. RAxML tree based on 955 *E. faecium* core genes in 1,644 clade A isolates (adapted from**
576 **Arredondo et al, 2020 (9)).** A: Nodes are colored to sources. Metadata blocks indicate the previously
577 defined sequence clusters (SC), the presence/absence of *ICEEfm1* and presence/absence of the *iol*
578 element. The arrow indicates the previous defined split of clade A1. B and C indicate the position of
579 the subtrees from panel B and C. B: Subtree of genetically closely related isolates where *ICEEfm1* is
580 present/absent. Nodes are colored to presence/absence of *ICEEfm1*. ESP: Spain; NLD: the
581 Netherlands; PRT: Portugal. C: Subtree of genetically closely related isolates where the *iol* element is
582 present/absent. Nodes are colored to presence/absence of *ICEEfm1*. Absence is indicated with green
583 and presence is indicated with yellow. NLD: the Netherlands; PRT: Portugal; SGP: Singapore.

584

585

586 **Fig. 2. Structural organization of the *iol* gene clusters of *Bacillus subtilis* and strain *E. faecium***
587 **E1504.** Blue arrows, *iol* genes that are present in both gene clusters; red arrows, *B. subtilis* *iol* genes
588 that are absent in *E. faecium*; orange arrow, the *E. faecium* putative transcriptional repressor; light
589 purple arrow, a transposase and green arrows, two additional genes absent in *B. subtilis*. The protein
590 functions encoded by the *iol* genes are provided in the table. The *B. subtilis* *iol* gene cluster has been
591 described by Yoshida *et al.* (32).

592

593 **Fig. 3. Growth curves of *E. faecium* to determine the ability to grow on inositol.** A: Overnight
594 cultures of E1679 (donor), E4658 (recipient), E7855 (transconjugant) were inoculated at an initial cell
595 density of OD₆₀₀ 0.0025 in M1 medium with 1% *myo*-inositol (M1-MI). B: Overnight cultures of wild-
596 type E1504 and E1504Δ*iolD* were inoculated at an initial cell density of OD₆₀₀ 0.0025 in M1 medium
597 with 1% *myo*-inositol (M1-MI) and for wild-type E1504 also in M1 medium in absence (M1) and
598 presence of 1% *D-chiro*-inositol (M1-DCI). Growth was measured every 15 minutes for 18 hours.

599

600 **Fig. 4. Transfer of ICEEfm1 from *E. faecium* E1679 to *E. faecium* E4658.** A: representative SYBR safe
601 stained PFGE gel of SmaI-digested chromosomal DNA of the donor strain (E1679; lane 1), the
602 recipient strain (E4658; lane 2) and the transconjugant (E7855; lane 3). The gel band that has shifted
603 in the recipient strain due to the insertion of ICEEfm1 is indicated by the white arrow. B: Southern
604 blot of the PFGE gel hybridized using an internal fragment of the *iol* gene cluster as probe.

605

606 **Fig. 5. qRT-PCR analysis of *lacl*, *iolD*, *iolA* and *sss* expression ratios.** *E. faecium* E1504 at OD₆₆₀ 0.3
607 grown in BHI, BHI-MI and M1-MI. The data from the qRT-PCR were normalized using *tufA* as an
608 internal standard. The differences in gene expression (log₂- transformed data) relative to growth in
609 BHI are shown (n=1).

610

611 **Fig. 6. *iolC* promoter mapping.** In red stop codon *lacl* and start codon *iolC* and transcription start
612 (+1). Putative -35 and -10 sequences are underlined, in blue putative transcriptional terminator of
613 *lacl*, in green putative binding site for *lacl* repressor.

614

615 **Fig. 7. Intestinal colonization.** Mice were orally inoculated with E1504 (blue dots) and E1504Δ*iolD*
616 mutant (red dots). A: Numbers of *E. faecium* E1504 and E1504Δ*iolD* were determined in fecal pellets
617 of mice at day 1, 3 and 10 after inoculation. B: After 10 days of colonization numbers of E1504 and
618 E1504Δ*iolD* were determined in the colon, cecum and ileum.

619

620 **Suppl. Fig. 1: Organization of the *iol* element encoded on ICEEfm1 in strain *E. faecium* E1679**
621 **(adapted from van Schaik et al. (18)).** The *iol* element is encoding for five genes of unknown function
622 (grey arrows) and the *iol* gene cluster, including the *iol* genes indicated in blue, a putative
623 transcriptional repressor (red), a transposase (yellow) and two other genes (green).

624

625 **Suppl. Fig. 2: Panaroo gene neighborhood analysis to determine the integration site of the *iol***
626 **element using strain *E. faecium* E7356 as reference.** A: Neighboring genes for E7356_0303 (light
627 blue), the first gene of the *iol* element as determined for 176/180 isolates. B: Neighboring genes for
628 E7356_0286 (red), the last gene of the *iol* element. Green box: presence of the gene; grey box:
629 absence of gene; blue box: presence of hypothetical gene different from E7356; orange box:
630 presence of a transposase not present in E7356; yellow: gene was not identified as neighboring gene
631 due to contig breaks in these isolates.

632

633 **Suppl. Fig 3: Genetic organization, MI catabolic pathway and functional activities of the *B. subtilis***
634 ***iol* genes (adapted from Yoshida et al., 2008 (32)).** A: *B. subtilis* *iol* genes proven to encode the
635 enzymes involved in the various reaction steps of the MI catabolic pathway are shown. Blue boxes
636 indicate *iol* genes also identified in the *E. faecium* *iol* gene cluster. B: Overview protein functions *iol*
637 genes. C: Overview of the compounds that are converted by the *Iol* enzymes

638

639 **Suppl. Fig. 4: Graphic summary of *LacI* protein BLAST result.**

640

641 **Suppl. Fig. 5. Structural organization of the *iol* gene cluster in strain *E. faecium* E1504.** A: Genomic
642 organization of the *iol* gene cluster, including the *iol* genes indicated in blue, a putative
643 transcriptional repressor (red), a transposase (yellow) and two other genes (green). Also indicated
644 are the predicted transcription terminators (RNAfold), PCR and primer sites for the determination of
645 transcriptional organization. Blue arrows indicate the primers that were used to synthesize cDNA and
646 that were used in combination with the upstream located primer in red for the amplification of the
647 intergenic region. B: PCR results for the 12 PCRs as indicated in panel A. As negative control PCR-2
648 was also performed on RT- cDNA. As positive control PCR-2 was performed on E1504 genomic DNA.
649 C: downstream *lacI* predicted terminator (RNAfold). D: downstream *pts* predicted terminator
650 (RNAfold).

