1 # Isolation and characterization of a nitrosoguanidine- induced *Enterococcus*

2 # *faecium* MTCC 5153 mutant defective in enterocin biosynthesis

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4 **Prakash M. Halami**

5 Department of Food Microbiology, Central Food Technological Research Institute,

6 Mysore- 570 020; CSIR, India.

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11 ***Running title: enterocin biosynthesis defective mutant***

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19 **Correspondence** *Prakash M. Halami; Food Microbiology Department, Central Food*

20 *Technological Research Institute, Mysore 570 020 India. Tel.:+ 91 821 2517 539; fax: +91 821*

21 *2517 233 E-mail address: prakashalami@cftri.res.in (PM Halami)*

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25 **Abstract**

26 Enterocin A (EntA) is a low molecular weight, heat-stable, chromosomally encoded class IIa
27 bacteriocin produced by several strains of *Enterococcus faecium*. In this study, mutated strain of
28 *E. faecium* MTCC 5153 was characterized for its sensitivity to EntA, immunity function and for
29 the production of induction factor. Nucleotide sequencing of the putative promoter of *ent*A
30 operon suggested point mutations upstream of *ent*A gene. The mutant was sensitive to several
31 class IIa bacteriocins and was found to adsorb 20% more bacteriocin compared to its wild-type
32 counterpart.

33 *Keywords:* Chemical mutagen; *Enterococcus faecium*; Enterocin A biosynthesis;
34 Induction factor; Immunity assay

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37 **1. Introduction**

38 Enterococci are the group of lactic acid bacteria (LAB) that have importance in food, 39 public health and medical microbiology. Many strains of enterococci produce bacteriocins, 40 commonly referred to as enterocins, which are diverse and have potential use as food 41 biopreservatives [5,12]. EnterocinA (EntA) represents an important class II enterocin of pediocin 42 family [5]. The *ent*A operon on the chromosome of *E. faecium* comprises of 10.5 Kb region that 43 is required for bacteriocin production, immunity and induction, transport, regulation etc [13].

44 EntA biosynthesis has been studied *in vivo* in *E. faecium* DPC1146 mutated by 45 transposon induced mutation or acridine orange treatment [13]. However, these mutants have 46 not been analyzed for their immunity function or sensitivity towards different bacteriocins. In 47 addition, sequences of the *ent*A promoter 500 bp upstream has been cloned for designing the 48 expression system [9]. Additionally, in the enterocin regulated promoter, involvement of direct 49 repeat sequences that are binding sites of the phosphorylated response regulator have been 50 identified [12]. However, there is a lack of knowledge on understanding the regulatory 51 mechanism of EntA synthesis and its immunity function *in vivo*. The objective of this study was 52 to characterize the promoter sequences of *ent*A operon in enterocin biosynthesis defective 53 mutant. The mutant defective in putative immunity (EntI) and induction factor (EntF) functions 54 was also characterized along with its sensitivity to various antimicrobial compounds.

55 **2. Materials and methods**

56 *2.1. Bacterial strains and culture conditions*

57 LAB cultures used in this study were *Enterococcus faecium* MTCC 5153, its mutant 58 derivative *Enterococcus faecium* ΔMTCC 5153, *Enterococcus faecium* BL (native enterocin 59 producer), *E. faecium* DPC 1146 and *Pediococcus acidilactici* PAC 1.0. *Listeria monocytogenes*

60 Scott-A was used as the bacteriocin indicator. All the LAB cultures were cultivated on MRS (Hi

61 Media, Mumbai, India) media at 37^{0}C under static condition and *L. monocytogenes* was grown in

62 BHI broth (Hi Media) at 37^{0}C under shaking (200 rpm). *Bacillus subtilis* ATCC6633 (subtilin

63 producer) and *Micrococcus luteus* ATCC9341 were grown in TY (1% each of tryptone, yeast

64 extract and NaCl) broth at 37^{0}C under shaking (200 rpm) or in TY-agar (1.2%) plates. All the

65 bacterial strains were stored at –20°C in 50% sterile glycerol until further use.

66 *2.2. Isolation and characterization of enterocin defective mutant*

67 A thick cell suspension of *E. faecium* MTCC 5153 (grown in BHI broth for 6 h) was

68 spread on MRS agar and a few drops of nitrosoguanidine (NTG, 10 mg ml^{-1}) (M/s Sigma;

69 Aldrich, USA), was spotted on the lawn and incubated at 37^{0}C for 20 h. Post incubation, isolated

70 colonies (ca. 5-7 nos.) that appeared in the mutagen diffused area were individually tested for the

71 production of enterocin by spotting the mutant colony on the lawn of the indicator, *L.*

72 *monocytogenes* Scott-A. The mutant of *E. faecium* MTCC 5153 was isolated based on its

73 inability to produce a zone of inhibition against the indicator. The authenticity of mutant was

74 confirmed by 16S rRNA gene sequencing.

75 *2.3. DNA sequence comparisons*

76 Standard protocols were followed for all molecular biology techniques [14]. Total DNA

77 from *E. faecium* MTCC 5153 was extracted as described by Mora et al. [11] and used as a

78 template for PCR amplification. The 16S rRNA gene was amplified as described by Halami et al.

79 [7]. PCR characterization of the partial (2.2 kb) *ent*A operon was accomplished by using the

80 primers Ent-F (5'ACAAAGTATTAGACAATTTCCA3') and Entin-R. (5'TTGGTATAGCTTC

81 GCTATCA3'). PCR amplification, conditions included 95°C for 5 min, followed by 35 cycles of

82 94°C for 40 sec, 55°C for 30 sec and 72°C for 3 min. For amplifying the putative promoter of

83 1.2 kb, Ent-F and Ent-R (5'TGATGCCGGTTTTCCTTGA)3' primers were used. PCR
84 conditions in this case were same as above except for 1.5 min during synthesis. The amplified
85 PCR product was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and
86 cloned into pTZ57R/T (MBI Fermentas, Lithuania) vector for sequencing. BLAST search of the
87 gene sequences obtained were compared to the non-redundant database [1]. The putative
88 promoter sequences were analyzed using the program TFSEARCH [8].

89 *2.4. Bacteriocin production and sensitivity*

90 The bacteriocin producing LAB cultures grown at 37°C for 18 h in MRS medium were
91 centrifuged to obtain the crude culture filtrate (CF). An aliquot of CF (50 ml) was extracted with
92 an equal volume of butanol, followed by lyophilizing the butanol extract and re-dissolving in 1
93 ml of water. It was filter sterilized (0.45μm) and used for evaluating bacteriocin activity against
94 *L. monocytogenes* Scott A. Similarly, activity of bacteriocin of *B. subtilis* ATCC6633 was tested
95 against *M. luteus* ATCC 9341. Sensitivity of *E. faecium* ΔMTCC 5153 and its wild type (WT)
96 counterpart was tested using bacteriocin preparations of *E. faecium* (DPC1146, BL and
97 MTCC5153) and *P. acidilactici* PAC1.0. The other antimicrobial compounds used in this study
98 were SDS, lysozyme, ampicillin, penicillin, cephalosporin (Sigma Chemicals, USA) and nisin
99 (ICN Biochemicals, USA; crude preparation 2% nisin). Minimum inhibitory concentration
100 (MIC) of antimicrobial compounds (AMC) against test cultures was studied in microtitre plate as
101 described previously [4]. MIC indicated the concentration of each AMC at which 50% reduction
102 in growth occurred.

103 *2.5. Immunity and induction assay*

104 For testing the immunity function, the filter sterilized preparation of different bacteriocins
105 adjusted to pH 6.5, was concentrated to 10 times by lyophilization. It was used to determine the

106 sensitivity of the indicator cultures by agar well diffusion assay [6]. Definite zone of inhibition
107 by the bacteriocin was considered to ascertain the loss of immunity function. For induction
108 assay, CF of MTCC 5153 grown for 15h was immobilized with Amberlite™ XAD™ absorbant
109 resin (Serva, Heidelberg, Germany) and the spent supernatant with a reduced antimicrobial
110 activity was used as a source of IF at 1% level (v/v). CF of *E. faecium* ΔMTCC5153 was also
111 prepared as a source of IF as described above. Test culture was grown for 15 h, harvested by
112 centrifugation and cell pellet obtained was washed several times with sterile water.
113 Subsequently, cells were suspended in sterile water and pour plated using MRS agar along with
114 CF (as a source of IF) of mutant and WT. After 15 h of growth, the plates were overlaid with
115 freshly grown indicator, *L. monocytogenes* Scott A. Upon the growth of indicator, size of zone of
116 inhibition produced by WT and mutant colonies were compared.
117 *2.6. Enterocin adsorption*
118 *E. faecium* MTCC 5153 and its mutant were grown in MRS broth (each 200 ml and 100
119 ml, respectively) for 15 h at 37°C. CF of WT (100 ml) was adjusted to pH 6.5 and adsorbed on
120 to heat-treated (70°C for 30 min) cells of the mutant. The WT culture formed the control for
121 comparing enterocin adsorption. The mixture was stirred at RT for 30 min and the enterocin-cell
122 complex was separated by centrifugation at 10, 000 rpm for 30 min at 4°C. Percent adsorption
123 was calculated based on enterocin activity before and after adsorption as described previously
124 [15]. Spot-on-lawn assay was performed for estimating the enterocin activity against Scott A and
125 expressed as AU ml^{-1} [7].
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129 **3. Results**

130 In order to provide a genetic basis for enterocin production by *E. faecium* MTCC 5153*,* an
131 enterocin defective mutant strain was produced using NTG. The mutant strain did not inhibit *L.*
132 *monocytogenes* Scott-A suggesting its inability to produce enterocin.

133

134 *3.1. Immunity and induction assay*

135 Mutant *E. faecium* (ΔMTCC5153) was sensitive to the bacteriocin produced by the WT
136 as well as EntA of DPC1146, suggesting loss of immunity function (Fig. 1a). Zone of inhibition
137 exhibited by ΔMTCC5153 was much larger (14 mm) as compared to Scott-A (10 mm). In the
138 enterocin induction assay, CF of WT induced enterocin production was similar to the parental
139 culture (zone size 6 mm). However, such induction was not observed when CF of mutant was
140 used (zone size 3 mm) indicating inability of mutant to synthesize active IF (Fig. 1b). However,
141 when only WT washed cells were plated, smaller zones were observed.

142

143 *3.2. DNA sequence comparisons*

144 Sequencing of the DNA fragment of the partial putative enterocin A promoter (1.2 kb) of
145 ΔMTCC5153 and its comparison to the WT indicated a few point mutations in the upstream
146 region of *ent*A gene (ATG ±0/1). These point mutations were G to A, T to A and A to G at -326,
147 -405 and -418 bp, respectively (Fig. 2). Computational analysis of the putative promoter region
148 of ΔMTCC5153 indicated its inability to bind the two heat-shock transcriptional factors (TF).
149 Interaction with one of the negative regulators at –326 region was also observed. The mutation
150 by NTG treatment probably is associated with affinity of these TF suggesting their possible role
151 of DNA-protein interaction [2]. Sequence analysis of 2.2 kb partial enterocin operons revealed

152 very few point mutations in the downstream region of the *ent*A gene with no changes in the
153 translated reading frame. The inability of mutant to drive the expression of putative *ent*A
154 promoter in the upstream region suggests the involvement of direct repeats in the regulated
155 promoters.
156
157 *3.3. Enterocin adsorption*
158 In order to evaluate the suitability of the mutant for enterocin concentration and
159 purification, WT and mutant cultures were subjected to enterocin adsorption assay. It was found
160 that at least 75% of the enterocin gets adsorbed onto the mutant strain compared to 60% on the
161 WT. The desorbed enterocin was of high titre compared to enterocin eluted from the host (Fig.
162 3). Similarly, the inoculum adjusted to pH 2.0 had maximum enterocin activity (AU ml^{-1}) of
163 1200 units compared to 804 units at pH 4.5. The difference in percent adsorption of indicator and
164 host clearly indicates the utility of the mutant for concentrating the bacteriocin.
165 *3.4. Bacteriocin production and sensitivity*
166 To study response of the mutant to cell wall inhibiting compounds, its sensitivity pattern
167 was evaluated. Interestingly, the mutant culture of *E. faecium* MTCC5153 was found to be 10
168 times more sensitive to class IIa bacteriocins compared to WT. Since the crude bacteriocin
169 preparation was used in the assay, MIC (μg ml^{-1}) values were found to be in the range of 0.5-1
170 for mutant and 4-8 for WT (Fig. 4). However, clinical antibiotics, nisin and subtilin did not affect
171 the growth of both cultures.
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175 **4. Discussion**

176 EntA, a broad spectrum anti-listerial bacteriocin, is reported from *E. faecium* isolated
177 from several sources [5,12]. EntA biosynthesis operon consists of ten genes comprising three
178 regulated promoters. The *ent*A promoter drives the expression of atleast 5-genes viz. *ent*A, *ent*I,
179 *ent*F, *ent*K and *ent*R [12,13]. Therefore the mutant obtained in this study was unable to produce
180 enterocin or exhibit immunity function or induce the enterocin production. The ΔMTCC5153
181 showed few interesting features with respect to its sensitivity to antimicrobial compounds.
182 Although immunity system of bacteriocin producing culture is highly specific to its cognate
183 bacteriocin [4], cross-sensitivity to the class II bacteriocins was observed in this study. These
184 results are in contrary to the observations made previously, no cross sensitivity of mutant to other
185 bacteriocins of pediocin family was seen [13].

186 Enterocin immunity represent subgroup IIA family of bacteriocin immunity, whose
187 crystals revealed that the protein folds into an anti-parallel form of helix bundle with a flexible
188 C-terminal in the half of cognate bacteriocin [10]. Diep et al, [3] demonstrated the involvement
189 of mannose phospho-transferase system (man-PTS) of susceptible cells as target/receptor. It
190 forms the complex between the immunity protein LciA and acts as a receptor that forms the
191 strong complex with the bacteriocin, thereby preventing cells from being killed. On the other
192 hand, plantaricin 423, shares a high sequence similarity with the pediocin PA-1 operon and no
193 cross-reactivity was recorded between their immunity proteins [16]. Bacteriocin adsorption on
194 the cell surface varies from 20 to 100% in either indicator or resistant bacteria. The adherence to
195 the cell surface was found to occur at random and not always at specific receptor sites [15]. In
196 the present study, higher adsorption of enterocin to mutant was observed as compared to WT.

197 Increase in percentage adsorption by mutant cells suggests indirect involvement of the immunity
198 protein.

199 In conclusion, this study indicates the utility of enterocin defective mutant for
200 ascertaining the sensitivity of highly diversified class II bacteriocins. Additionally, this mutant
201 can also be exploited for studying *in vivo* functions of bacteriocin-immunity proteins. Added to
202 this, the strength of the mutated promoter of *ent*A operon can also be analyzed.

203

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209 **References**

210 [1] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman,
211 D.J. (1997) Gapped BLAST and PSIBLAST, a new generation of protein database search
212 programs. Nucl. Acids Res. 25, 3389–3402.

213 [2] Bouhouche, N., Syvanen M., Kado, C.I. (2000) The origin of prokaryotic C2H2 zinc
214 finger regulators, Trends Microbiol. 8**,** 77–81.

215 [3] Diep, D.B., Skaugen, M., Salehian, Z., Holo, H., Nes, I.F. (2007) Common mechanisms
216 of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci.
217 104, 2384-2389.

218 [4] Fimland, G., Eijsink, V.G.H., Nissen-Meyer, J. (2002) Comparative studies of immunity
219 proteins of pediocin-like bacteriocins. Microbiology. 148, 3661-3670.

220 [5] Franz, C.M.A.P., van Belkum, M.J., Holzapfel, W.H., Abriouel, H., Galvez, A. (2007)
221 Diversity of enterococcal bacteriocins and their grouping in a new classification scheme.
222 FEMS Microbiol. Rev. 31, 293-310.
223 [6] Geis, A., Singh, J., Teuber, M. (1983) Potential of lactic streptococci to produce
224 bacteriocin. Appl. Environ. Microbiol. 45, 205-211.
225 [7] Halami, P.M., Ramesh, A., Chandrashekar, A. (2005) Fermenting cucumber, a potential
226 source for the isolation of pediocin-type bacteriocin producers. World J. Microbiol.
227 Biotechnol. 21, 1351-1358**.**
228 [8] Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A., Kel, O., Ignatieva, E.,
229 Ananko, E., Podkolodnaya, O., Kolpakov, F., Podkolodny, N., Kolchanov, N. (1998)
230 Databases on Transcriptional Regulation: TRANSFAC, TRRD, and COMPEL. Nucl.
231 Acids Res. 26, 364-370.
232 [9] Hickey, R.M., Twomey, D.P., Ross, R.P., Hill, C. (2003) Potential of the enterocin
233 regulatory system to control expression of heterologous genes in *Enterococcus.* J. Appl.
234 Microbiol. 95, 390–397.
235 [10] Johnsen, L., Dalhus, B., Leiros, I., Nissen-Meyer, J. (2005) 1.6-Å Crystal structure of
236 EntA-im: a bacterial immunity protein conferring immunity to the antimicrobial activity
237 of the pediocin-like bacteriocin enterocin A. J. Biol. Chem. 280, 19045–19050.
238 [11] Mora, D., Fortina, M. G., Parini, C., Ricci, G., Daffonchio, D., Manachini, P. L. (2000)
239 Genomic subpopulations within the species *Pediococcus acidilactici* detected by
240 multilocus typing analysis: relationship between pediocin AcH/PA-1 producing and
241 non-producing strains. Microbiology. 146, 2027-2038.

242 [12] Nes, I. F., Diep, D. B., Holo, H. (2007) Bacteriocin diversity in *Streptococcus* and
243 *Enterococcus*. J. Bacteriol. 189**,** 1189-1198.
244 [13] O'Keffe, T., Hill, C., Ross, R.P. (1999) Characterization and heterologous expression of
245 the genes encoding enterocin A production, immunity, and regulation in *Enterococcus*
246 *faecium* DPC 1146. Appl. Environ. Microbiol. 65, 1506-1515.
247 [14] Sambrook, J., Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd
248 edition, Cold Spring Harbor Laboratory Press.
249 [15] Todorov, S.D., Powell, J.E., Meincken, M., Witthuhn, R.C., Dicks, L.M.T. (2007)
250 Factors affecting the adsorption of *Lactobacillus plantarum* bacteriocin bacST8KF to
251 *Enterococcus faecalis* and *Listeria innocua*. Int. J. Dairy Technol. 60, 221-227.
252 [16] Van Reenen, C.A., Van Zyl, W.H., Dicks, L.M.T. (2006) Expression of the immunity
253 protein of plantaricin 423, produced by *Lactobacillus plantarum* 423, and analysis of the
254 plasmid encoding the bacteriocin. Appl. Environ. Microbiol. 72, 7644–7651.
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257 **Legend of figures**

258 **Figure 1.** Characterization of *E. faecium* ΔMTCC 5153 for enterocin production, immunity and
259 induction of enterocin. a) Agar well diffusion assay to study bacteriocin production and
260 immunity function of *E. faecium* ΔMTCC5153. 5153, *E. faecium* MTCC 5153; Δ5153, *E.*
261 *faecium* ΔMTCC 5153; DPC, *E. faecium* DPC1146. P, test CF of bacterial strains; I, bacterial
262 strains used as an indicator, Scott-A, *L. monocytogenes* Scott-A used as a control. b) Induction of
263 enterocin production by the CF of WT and mutant.

264

265 **Figure 2.** Comparison of putative promoter sequences of enterocin A biosynthesis genes among
266 WT and mutant (M) strain of *E. faecium* MTC5153. DPC, *E. faecium* DPC1146 sequences used
267 for comparison. Shaded areas are changes in nucleotide observed in the upstream sequences of
268 enterocin operon.

269

270 **Figure 3**. Comparison of enterocin A adsorption to WT and mutant strains of *E. faecium* MTCC
271 5153. Total CF activity (▩) obtained upon extraction in pH 2.0. Residual bacteriocin and cell
272 desorbed bacteriocin in WT (|||||) as well as M (▩) cultures respectively, is shown. The data
273 presented representing the average values of two independent experiments with a standard
274 deviation indicated by error bars.

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276 **Figure 4.** Response of antimicrobial compounds to WT ( ) and mutant ( ) of *E. faecium*
277 MTCC 5153.

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279 **Figure 1**

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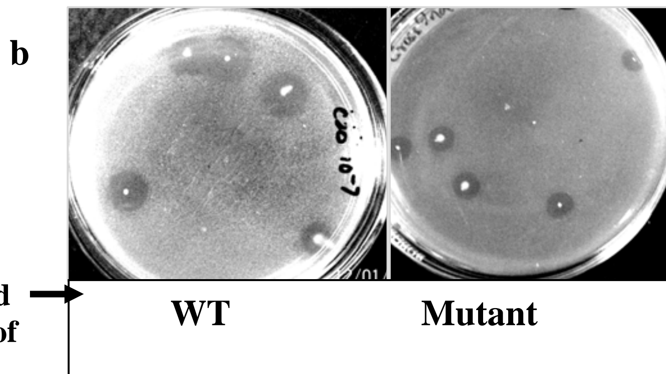

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295 **Figure 2.**

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                -418                 -405                                                   -326
DPC  AAGAATTAGT  CGAAAGCTGG  AAAAAATAAA  ATTTGGAAGC  TCCATGTTCG
WT   AAGAATTAGT  CGAAAGCTGG  AAAAAATAAA  ATTTGGAAGC  TCCATGTTCG
M    AAGAGTTAGT  CGAAAGCAGG  AAAAAATAAA  ATTTGGAAGC  TCCATATTCG
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300 **Figure 3.**

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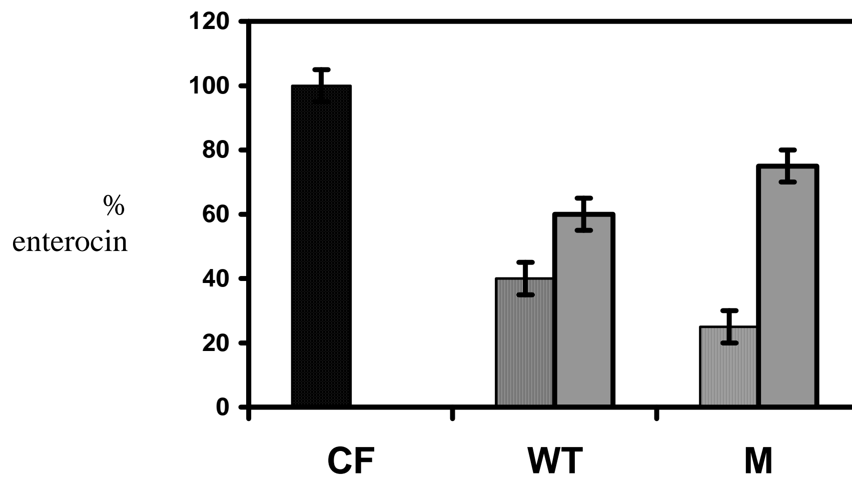

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303 **Figure 4.**

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