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**Transduction of *stx2a* mediated by phage (Φ11-3088) from *Escherichia coli***

**O104:H4 *in vitro* and *in situ* during sprouting of mung beans**

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14     **Abstract**

15     *Escherichia coli* O104:H4 strain 11-3088 encoding Stx2a is epidemiologically related to

16     the foodborne outbreak associated with sprouts in Germany, 2011. Sprouting provides

17     suitable conditions for bacterial growth and may lead to transduction of non-pathogenic

18     strains of *E. coli* with Stx phages. Although transduction of *E. coli* by Stx phages in food

19     has been documented, data on the phages from *E. coli* O104:H4 is limited. This study

20     determined the host range of the bacteriophage Φ11-3088 from *E. coli* O104:H4 using *E.*

21     *coli* O104:H4 Δ*stx2::gfp::amp^r* and demonstrated phage transduction during sprouting.

22     The Φ11-3088Δ*stx* transduced 5/45 strains, including generic *E. coli*, *pap*-positive *E. coli*

23     O103:H2, ETEC, and *S. sonnei*. The expression level of Φ11-3088Δ*stx* differed among

24     lysogens upon induction. Of the 3 highly induced lysogens, the lytic cycle was induced in

25     *E. coli* O104:H4Δ*stx2::gfp::amp^r* and O103:H2 but not in *S. sonnei*. *E. coli* DH5α was the

26     only strain susceptible to lytic infection by Φ11-3088Δ*stx*. To explore the effect of drying

27     and rehydration during seed storage and sprouting on phage induction and transduction,

28     mung beans inoculated with the phage donor *E. coli* O104:H4Δ*stx2::gfp::amp^r* (8 log

29     CFU/g) were dried, rehydrated, and incubated with the phage recipient *E. coli* DH5α (7 log

30     CFU/g) for 96 h. Sprouted seeds harbored about 3 log CFU/g of putative lysogens that

31     acquired ampicillin resistance. At the end of sprouting, 71% of putative lysogens encoded

32     *gfp*, confirming phage transduction. Overall, *stx* transfer by phages may increase the cell

33     counts of STEC during sprouting by converting generic *E. coli* to STEC.

34     **Keywords**

35     *E. coli* O104:H4, Stx2 phage transduction, virulence recombination, sprouts safety

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## 1. Introduction

Shiga toxin producing *E. coli* (STEC) are significant contributors to foodborne illness and mortality (Crim et al., 2015; Karmali, 2018). Shiga toxins (Stx) are the major virulence factor of STEC. Stx arrest ribosomal protein biosynthesis and cause hemolysis and kidney failure (Trachtman et al., 2012) but may also cause neurological symptoms. The *stx* genes are coded on the late region of lambdoid prophages (Herold et al., 2004). The expression of *stx* is repressed by the phage late gene repressor CI unless DNA damaging agents, such as hydrogen peroxide, mitomycin C or antibiotics, activate expression of late phage genes and *stx* (Fang et al., 2017; Johnson et al., 1981; Kimmitt et al., 2000). DNA damage induces the SOS response, which induces the prophages, followed by the production of toxin and phages through the lytic cycle (Kimmitt et al., 2000; Rozanov et al., 1998). Because certain antibiotics induce Stx phages, their use to treat STEC infection can exacerbate clinical symptoms by stimulation of the Stx production.

If progeny phages carrying *stx* transduce *E. coli*, commensal and diverse pathogenic *E. coli* including enterotoxigenic and enteroaggregative *E. coli* are converted to STEC (Beutin and Martin, 2012; Iversen et al., 2015; Nyholm et al., 2015). A hybrid strain of enteroaggregative-haemorrhagic *E. coli* (EAHEC) O104:H4 that encodes both *stx2a* and *agg* coding for enteroaggregative fimbriae, caused 4,000 cases and 50 deaths in Germany in 2011, making it one of the world's largest outbreaks of STEC (Beutin and Martin, 2012). The combination of virulence factors suggests that the parental strain of *E. coli* O104:H4 has acquired *stx2a* by phage transduction (Grad et al., 2013).

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58    The outbreaks caused by *E. coli* O104:H4 in 2011 in Germany and France were linked to

59    the consumption of contaminated fenugreek sprouts (Beutin and Martin, 2012). Enteric

60    pathogens including *E. coli* survive for long periods of time during dry storage of seeds

61    (Beuchat and Scouten, 2002). Moreover, the conditions for seed germination also provide

62    ideal conditions for bacterial growth, which can also increase the occurrence of foodborne

63    pathogens on sprouts. Several studies demonstrated that growth of STEC in food may result

64    in transduction of non-pathogenic *E. coli* with Stx phages but these studies used STEC

65    O157:H7 (Imamovic et al., 2009; Nyambe et al., 2017). Stx phages of EAHEC O104:H4

66    differ from Stx phages in *E. coli* O157:H7 with respect to the modules for DNA replication,

67    super-infection immunity, and tail fiber proteins (Beutin et al., 2012). The tail proteins

68    determine the host range of phages (Chatterjee and Rothenberg, 2012; Letellier et al., 2004),

69    therefore, the heterogeneity of the tail proteins also impacts the host range of phages. The

70    information of the host range of Stx phages from EAHEC O104:H4 is not as extensively

71    studied as the Stx phages from *E. coli* O157:H7. Different Stx prophages also differ with

72    respect to the environmental stressor that converts temperate phages to the lytic cycle

73    (Zhang et al., 2018). The conditions for phage induction together with the host range of

74    phages determines the ability of phages to spread virulence genes. Past studies

75    demonstrated that drying induces the expression of *stx-* encoding prophages upon

76    rehydration (Fang et al., 2020), which implies conditions for seeds germination may result

77    in transduction of non-pathogenic strains of *E. coli*, thus increasing the pathogen load on

78    sprouts.. However, data on the *stx*-encoding phage transduction during sprout production

79    is unavailable.

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80  Therefore, this study aimed to investigate the host specificity of Stx phage Φ11-3088 from

81  EAHEC O104:H4 and the transduction of non-pathogenic strains of *E. coli* by Φ11-3088

82  during seed germination. To avoid the generation of novel strains of STEC, the *stx2a*-

83  encoding Φ11-3088 from *E. coli* O104:H4 11-3088 was modified to replace *stx2a* with a

84  cassette encoding green fluorescent protein (*gfp*) and ampicillin resistance (*amp$^r$*) (Fang et

85  al., 2017).

86  **2.  Material and Methods**

87  **2.1. Bacterial strains and culture condition**

88  Bacterial strains used in this study are listed in Tables 1. *E. coli* O104:H4 11-3088 (Genome

89  accession number: LECH00000000) was isolated from a patient who was infected in 2011

90  in Germany and diagnosed with HUS in Canada (Liu et al., 2015). In the strain *E. coli*

91  O104:H4Δ*stx2::gfp::amp$^r$*, *stx2a* was replaced with *gfp* and *amp$^r$* (Fang et al., 2017). Φ11-

92  3088 and Φ11-3088Δ*stx2* refer to the phage produced by *E. coli* O104:H4 11-3088 and *E.*

93  *coli* O104:H4Δ*stx2::gfp::amp$^r$*, respectively. *E. coli* DH5α was used as a positive control

94  for *in vitro* lysogenic and lytic infection by Φ11-3088Δ*stx2*. Pathogenic bacteria included

95  6 strains of enterohemorrhagic *E. coli*, 2 strains of uropathogenic *E. coli* (UPEC), 5 strains

96  of enterotoxigenic *E. coli* (ETEC) and 2 strains of *Shigella*. ETEC strains were provided

97  by the Reference Laboratory for *E. coli* (ECL) of the Université de Montréal, Québec,

98  Canada. Non-pathogenic strains of *E. coli* included 14 isolates of *E. coli* from humans, 14

99  isolates of *E. coli* from the rectum or vagina of dairy cows (Dlusskaya et al., 2011; Wang

100 et al., 2013), and 11 strains of *E. coli* from a beef processing plant (Aslam et al., 2004).

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101 Strains of *E. coli* were aerobically incubated at 37 °C in Luria-Bertani (LB) broth and

102 strains of *E. coli* termed *Shigella* were incubated at the same conditions in Brain Heart

103 Infusion (BHI) (BD, Mississauga, CA). *E. coli* DH5α pJIR750ai carries the

104 chloramphenicol resistance gene on the plasmid pJIR750ai; thus the chloramphenicol

105 resistance was used as a selective marker for *E. coli* DH5α pJIR750ai. Ampicillin with a

106 final concentration of 100 mg/L or/and chloramphenicol with a final concentration of 30

107 mg/L were added into the nutrient media to select the antibiotic resistant cells.

108 **2.2. Preparation of phage filtrates**

109 *E. coli* O104:H4Δ*stx2::gfp::amp$^r$* was used as a donor of Φ11-3088Δ*stx2*. Phage filtrates

110 were prepared as described (Iversen et al., 2015). In short, *E. coli*

111 O104:H4Δ*stx2::gfp::amp$^r$* grown in LB broth with 5 mM $CaCl_2$ were harvested at the

112 exponential phase of growth ($OD_{600nm}$ 0.4 to 0.6) and induced with 0.5 mg/L mitomycin C,

113 followed by incubation at 37 °C overnight. Cells were removed by centrifugation and the

114 supernatant was filtered through 0.22 µm filters (Fisher Scientific, Ottawa, ON, CA). To

115 remove colicins, trypsin (Sigma-Aldrich, St Louis, MO, USA) was added to a

116 concentration of 0.1 g/L to the phage filtrate, followed by the incubation for 1 h at 37 °C.

117 The phage titer was determined by a plaque assay using *E. coli* DH5α as a sensitive

118 recipient strain. A mixture of 100 µL of exponential phase cultures of *E. coli* DH5α and

119 900 µL of phage filtrates was incubated at 37 °C for 30 min without agitation. After

120 incubation, the mixture was mixed with 3 mL of soft LB agar with 0.7 % agar and then

121 poured onto the LB agar containing 10 mM $CaCl_2$, followed by incubation at 37 °C for 18

122 h.

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123 **2.3. Lytic and lysogenic infection by Φ11-3088Δ*stx2***

124 The ability of *E. coli* to form lysogens and plaques was used to indicate the lysogenic and

125 lytic infection by Φ11-3088Δ*stx2,* respectively. Lysogenic infection was determined as

126 described by Schmidt et al. (1999). Acquisition of ampicillin resistance from *amp$^r$* via

127 Φ11-3088Δ*stx2* was used to select for cells that were lysogenized by Φ11-3088Δ*stx2*. To

128 avoid false positive results, all recipient strains were first plated on media containing

129 ampicillin to exclude false positive results caused by the native resistance to ampicillin.

130 Ampicillin resistant *E. coli* (Table 1) were not used in the assay for lysogenic infection. A

131 total of 10 pathogenic bacteria and 35 generic *E. coli* including *E. coli* DH5α were screened

132 as hosts for Φ11-3088Δ*stx2*. Exponential-phase cultures of each strain were mixed with

133 Φ11-3088Δ*stx2* filtrate in a 1:1 ratio, followed by incubation for 4 h at 37 °C (Schmidt et

134 al., 1999). Then, ampicillin (100 mg/L) was added to the culture, followed by incubation

135 for 24 to 48 h at 37 °C and 200 rpm agitation. After incubation, bacteria were collected by

136 centrifugation and plated onto LB agar containing ampicillin (LB-Amp). The presence of

137 Φ11-3088Δ*stx2* in colonies on LB-Amp plates was confirmed by PCR using the *gfp*-

138 specific primers F: TCCTGGTCGAGCTGGACG; R: TGGAGTTCGTGACCGCCG.

139 Lytic infection was determined by spot agar assay using 15 strains, including 5 strains that

140 were lysogenized by Φ11-3088Δ*stx2*, and the 10 ampicillin resistant strains that were

141 excluded from lysogenic infection (Iversen et al., 2015). Stationary phase cultures of each

142 strain (100 µL) were mixed with 3 mL of 0.7 % LB agar and then poured onto a standard

143 LB agar plate. After the solidification of the top layer of the agar, 10 µL of phage filtrates

144 were spotted on the top of the agar. Formation of clear zones on a bacterial lawn after the

145  incubation at 37°C for 18 h indicated lytic infections by Φ11-3088Δ*stx2*. The lysogenic

146  and lytic infections were repeated three times for each strain.

**2.4. Growth curve of lysogens after induction by mitomycin C**

148  Cell densities of six Φ11-3088Δ*stx2* encoding *E. coli* during incubation in the presence of

149  mitomycin C were measured by the absorbance of optical density at 600 nm ($OD_{600}$).

150  Bacterial culture of each strain harvested at $OD_{600}$ of 0.5-0.6 was suspended in LB or BHI

151  broth with and without mitomycin C. Nutrient media without mitomycin C served as blanks.

152  Two hundred microliter of each culture was transferred into the 96 microtiter plates in

153  duplicate. Plates were covered with optical films (Applied Biosystems™MicroAmp™,

154  Fisher scientific) to prevent water evaporation. Covering films were loosely sealed on the

155  edges of the plates to allow air access during incubation in the spectrophotometer at 37 °C

156  (Varioskan, Thermo Scientific). During incubation, plates were shaken and measured

157  every 20 min for 600 min. The values of $OD_{600}$ were corrected by subtracting the $OD_{600}$ of

158  the blank.

**2.5. Flow cytometric quantification of GFP fluorescence and forward light scatter**
    **(FSC)**

161  The expression of *gfp* in six Φ11-3088Δ*stx2*-encoding strains including *E. coli* DH5α,

162  *E. coli* O103:H2 PARC 444, ETEC ATCC 31618, the cow isolate *E. coli* FUA1043, *S.*

163  *sonnei* ATCC 25391, and *E. coli* O104:H4Δ*stx2::gfp::amp^r* were quantified by flow

164  cytometry (Fang et al., 2017). GFP-positive cells in the population of lysogens were

165  compared with *E. coli* O104:H4Δ*stx2::gfp::amp^r*. Exponential-phase cultures of *E. coli*

166  and *S. sonnei* were incubated with the addition of mitomycin C (0.5 mg/L) for 3 h to induce

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167   the prophage. Bacterial cultures incubated in the absence of mitomycin C served as non-

168   induced controls. The GFP fluorescence and forward-scattered light (FSC) were quantified

169   by flow cytometry immediately after mitomycin C induction. Bacterial culture with a

170   volume of 200 μL was diluted with 1 mL of 0.85% NaCl (Fisher Scientific) and further

171   diluted with FACS buffer (1% PBS, 2% fetal calf serum, 0.02% sodium azide) to maintain

172   the running speeds to no more than 3000 events per sec. Flow cytometry was performed

173   using a BD LSR-Fortessa X20 (BD Biosciences, San Jose, CA) equipped with a 488 nm

174   excitation from a blue air laser at 50 mW to excite green fluorescence (530±30 nm). Sample

175   injection and acquisition were started simultaneously and stopped when about 10,000

176   events were collected. FCS files were extracted from FACSDiva 8 software and analyzed

177   by FlowJo software (Tree Star, Ashland, USA). FSC is proportionally increased with cell

178   size and thus used to measure the cell volume (Koch et al., 1996). The gating of GFP

179   fluorescence and FSC was manually set to include more than 97% of the cells in control

180   samples as normal size and GFP negative. Four subpopulations were divided by two

181   reference lines, including GFP positive and elongated cell (GFP+, FSC+), GFP positive

182   and regular-sized cell (GFP+, FSC-), GFP negative elongated cell (GFP-, FSC+), and GFP

183   negative and regular-sized cell (GFP-, FSC-).

184   **2.6. Transduction of Stx phage during sprouts germination**

185   To investigate the transduction of *stx2*-encoding phages during seed germination, the phage

186   donor and recipient *E. coli* O104:H4Δ*stx2:gfp:amp$^r$* and *E. coli* DH5α pJIR750ai,

187   respectively, were inoculated on mung beans obtained in a local supermarket. Strains of *E.*

188   *coli* were harvested after 18-20 h of incubation in LB broth. A volume of 100 μL of the

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189    culture was plated onto the surface of LB agar and incubated overnight at 37 °C. Cells were

190    removed from the surface with 1 mL of 0.85% NaCl (Fisher Scientific, Ottawa, Ontario).

191    The cell count of the resulting cell suspensions was 10-11 $\log_{10}$(CFU/mL). Mung beans

192    were decontaminated with 0.2 % (v/v) sodium chlorine (Sigma-Aldrich) in water for 15

193    min, followed by washing twice with sterilized water and air-dried in a biosafety cabinet

194    (Fang et al., 2021). Ten grams of mung beans were inoculated with 1 mL of a cell

195    suspension of *E. coli* O104:H4Δ*stx2:gfp:amp^r*. Inoculated beans were air-dried in a

196    biosafety cabinet for 2 h and then stored in desiccators to keep the beans at a dry state until

197    use.

198    To germinate the inoculated mung beans, they were transferred into a 0.22 μm Millipore®

199    StericupTM filtration unit (Fisher Scientific), rehydrated with 10 mL sterilized water for 3

200    to 4 h, and incubated with the addition of *E. coli* DH5α pJIR750ai as phage recipient at 18-

201    20 °C for 96 h. Rehydrated beans were sampled after the addition of *E. coli* DH5α

202    pJIR750ai at 0 h; sprouts were sampled after 24, 48, 72, and 96 h of incubation. Bacteria

203    on the beans (2 kernels of 0.1-0.15 g) or sprouts (2 pieces of 0.1-0.35 g) were removed by

204    vortexing with 1 mL of 0.1% peptone water. The cell suspensions were serially diluted

205    with 1 mL of 0.1% peptone water (BD, Fisher Scientific), and plated onto LB agar, LB-

206    Amp, LB agar containing chloramphenicol plates (LB-Chl), and LB agar containing both

207    antibiotics (LB-Amp-Chl) to select for antibiotic-resistant colonies representing the donor,

208    the recipient, and lysogenized recipients, respectively. The Φ11-3088Δ*stx2* transduction

209    during seed germination was repeated in three independent experiments. The presence of

210    Φ11-3088Δ*stx2* prophage in colonies on the LB-Amp-Cm plates was confirmed with PCR

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211    amplification using *gfp*-specific primers described in Section 2.3. In addition, plasmids

212    were extracted from all *gfp*-positive strains using the plasmid DNA isolation GeneJET kit

213    (Thermo Fisher Scientific, Ottawa, ON, CA). The size of the plasmid was compared with

214    pJIR750ai from *E. coli* DH5α by gel electrophoresis (Bio-Rad, Mississauga, ON).

215    **2.7. Statistical analysis**

216    Flow cytometry data and cell counts were reported as average values of three independent

217    experiments. Statistical differences of the same sub-populations among different strains

218    and cell counts on the same antibiotic selective media among different days were analyzed

219    by one-way analysis of variance (ANOVA). Statistical differences were determined by

220    LSD test with an error probability of 5% using SPASS 21.0 (SPSS Inc., Chicago, IL, USA).

221    **3.    Results**

222    **3.1. Host range of Φ11-3088Δ*stx2***

223    To investigate the host range of Φ11-3088, 45 strains of *E. coli* (Tables 1&2) were each

224    exposed to about $10^4$ PFU/mL of Φ11-3088Δ*stx*. Five strains of these strains including *E.*

225    *coli* DN5α, *E. coli* O103:H2 PARC 444, *E. coli* ATCC 31618, *E. coli* FUA 1043 and *S.*

226    *sonnei* ATCC 25391 were lysogenized by Φ11-3088Δ*stx2* and acquired *amp[r]* as well as

227    *gfp* (Table 2). Several strains of *E. coli* were resistant to ampicillin; thus lysogenic infection

228    could not be determined (Table 2). Strains of *E. coli* containing *stx*, *eae*, or both were not

229    susceptible to Φ11-3088Δ*stx2*. Non-pathogenic strains of *E. coli* were not susceptible to

230    lysogenic infection by Φ11-3088Δ*stx2*, except for *E. coli* FUA1043. A total of 15 strains

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231 were tested for lytic infection; however, only *E. coli* DH5α, which lacks *recA* (Phue et al.,

232 2008), was susceptible to lytic infection.

**3.2. Growth curves of lysogens of Φ11-3088Δ*stx* in response to mitomycin C**

234 To determine whether the addition of mitomycin C induces the lytic cycle of the

235 Φ11-3088Δ*stx*2, growth curves of five lysogenized strains and *E. coli*

236 O104:H4Δ*stx2::gfp::amp$^r$* were determined (Fig. 1). The growth curves of *E. coli* O103:H2

237 PARC444 exhibited a similar pattern with that of *E. coli* O104:H4Δ*stx2::gfp::amp$^r$* (Fig.

238 1A and 1B). Different from *E. coli* O104:H4Δ*stx2::gfp::amp$^r$*, the growth curves, growth

239 rate, and maximum cell density of *S. sonnei* ATCC 25391 were similar with or without the

240 treatment with mitomycin C (Fig. 1C). For other strains of *E. coli*, mitomycin C arrested

241 growth but lysis of cells was not observed (Fig. 1D – 1F).

**3.3. Expression of *gfp* in different lysogens upon induction with mitomycin C**

243 Mitomycin C induces the SOS response in *E. coli* and results in the expression of Stx2

244 prophages and cell filamentation (Fang et al., 2017). Cell filamentation and induction of

245 Φ11-3088Δ*stx*2 in lysogens were quantified by the FSC and fluorescence of GFP,

246 respectively, using flow cytometry (Fig. 2). Mitomycin C induced >46 % cells of *E. coli*

247 O103:H2 PARC444 and *S. sonnei* ATCC 25391 expressing GFP, which was similar to *E.*

248 *coli* O104:H4Δ*stx2::gfp::amp$^r$* and significantly greater than the other three lysogens. In

249 populations of ETEC ATCC 31618, *E. coli* DH5α and *E. coli* FUA 1043, less than 20% of

250 cells expressed GFP after induction. Of the three strains that highly expressed GFP, about

251 10 % of cells were filamented. In populations of the three strains that did not highly express

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252 GFP, less than 1.5 % of cells were filamented (Fig. 2A). Low levels of GFP fluorescence

253 and filamentation were also detected in non-induced controls (Fig. 2B).

254 **3.4. Transduction of Stx phage during sprouting**

255 Desiccation followed by rehydration induced the expression of Stx prophages in *E. coli*

256 (Fang et al., 2020). Sprouting may thus support phage transduction to non-pathogenic

257 strains of *E. coli* that are present on the seeds. To investigate the links between drying and

258 rehydration on the effect of induction and transduction of Stx phages, mung beans were

259 inoculated with *E. coli* O104:H4Δ*stx2a::gfp::amp^r*, dried, rehydrated and co-incubated

260 with *E. coli* DH5α pJIR750ai during sprouting. Cells of *E. coli* DH5α that were lysogenized

261 with Φ11-3088 were quantified by plating on LB-Amp-Chl (Fig 3). The microbial counts

262 of chlorine-treated mung beans and their sprouts were below the detection limit of 2.3

263 $\log_{10}$(CFU/g). During sprouting, the cell counts of *E. coli* O104:H4Δ*stx2::gfp::amp^r* and

264 *E. coli* DH5α pJIR750ai were higher than 8 $\log_{10}$(CFU/g) (Fig 3). Ampicillin and

265 chloramphenicol resistant *E. coli* on sprouts were considered as tentative lysogens of

266 Φ11-3088Δ*stx2*. The number of tentative lysogens increased from about 1.18±1.67

267 $\log_{10}$(CFU/g) at 0 h of sprouting to 3.88±0.76 $\log_{10}$(CFU/g) at 96 h of sprouting (Fig. 3).

268 The presence of Φ11-3088Δ*stx2* and the plasmid pJIR750ai was verified in 92 putative

269 lysogens that were isolated from each of the three biological replicates. The simultaneous

270 presence of *gfp* and pJIR750ai, which is indicative of phage transduction, was confirmed

271 for 34 of the 92 isolates (Table 3). At the end of sprouting, 71% of putative lysogens were

272 PCR-positive for *gfp*, all of which tested for plasmid confirmation carried pJIR750ai (Table

273 3).

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274 **4. Discussion**

275 Sprouted seeds are often consumed raw and have been linked to numerous foodborne

276 outbreaks in the past 20 years, making them a significant food safety concern (Dechet et

277 al., 2014). Several international sprouts-associated outbreaks have been traced back to

278 single seed supplies, including the outbreak caused by EAHEC O104:H4 in Germany and

279 other European countries in 2011 (Beutin and Martin, 2012; Mahon et al., 1997; Taormina

280 et al., 1999). Only few enteroaggregative *E. coli* (EAEC) are known to carry *stx2a* (Iyoda

281 et al., 2000; Morabito et al., 1998), resulting in a novel pathotype, EAHEC (Beutin and

282 Martin, 2012). Transduction of EAEC with Stx2 phages has previously been reported *in*

283 *vitro* (Beutin and Martin, 2012). This study provides a proof of concept that STEC survives

284 on dry seeds, resulting in transduction of Stx phages to non-pathogenic *E. coli* during

285 sprouting.

286 **4.1. Host range of Φ11-3088 of *E. coli* O104:H4**

287 Stx phages have variable host ranges, depending on the genetic makeups of both phages

288 and recipient bacteria (Beutin et al., 2012; Ranieri et al., 2014). With exception of *E. coli*

289 DH5α, strains of *E. coli* were not susceptible to lytic infection by Φ11-3088 and Φ13374

290 from *E. coli* O104:H4 (this study, Beutin et al., 2012). Genetic modification of the tail

291 spike proteins shifts the host range of phages (Holmes, 2009; Pepin et al., 2010). The host

292 specificity of the λ phage depends on the binding specificity between the membrane

293 receptor LamB and the tail proteins GpJ (Chatterjee and Rothenberg, 2012; Wang et al.,

294 2000). Adsorption of the Stx phages is also well described for the membrane protein YaeT,

295 which is recognized by the tail spike protein associated with short-tail morphology (Smith

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296    et al., 2007). The genome of Φ11-3088 shares 99.8% homology with Φ13374 (this study,

297    data not shown), but both phages differ from Φ933W (65% DNA homology) (Ranieri et

298    al., 2014). The host spectrum of Φ11-3088 appears to be narrower than the host spectrum

299    of Φ933W (Gamage et al., 2004). The unique traits of the tail gene cluster in Stx phages

300    from *E. coli* O104:H4 might contribute to this narrow host spectrum when compared to the

301    *E. coli* O157-derived phages, including Φ734, Φ24B, Φ933W, ΦA557, and Φ3538

302    (Gamage et al., 2004; Herold et al., 2004; Imamovic et al., 2009; McCarthy et al., 2002;

303    Schmidt et al., 1999). Of note, Φ11-3088 transduced *S. sonnei*. Clinical isolates of *Shigella*

304    generally carry Stx1 (Beutin et al., 1999; Strauch et al., 2001); however, Stx2 prophages

305    encoding lysogeny of *Shigella* species was reported after *in vivo* transduction (McCarthy

306    et al., 2002; Schmidt et al., 1999; Tozzoli et al., 2014).

307    **4.2. Induction of *stx2a* prophages in *E. coli* O104:H4**

308    The induction of the lytic cycle, which is associated with production of Stx, genetically

309    links to the host recombinase system (Grzegorz et al., 2012). *E. coli* DH5α is deficient in

310    the major recombinase RecA, which makes it more susceptible to lytic infection by Stx

311    phages from a broad range of hosts (Muniesa et al., 2004; this study). The regulation of Stx

312    production depends on the genotype of phage and host (De Sablet et al., 2008; Zhang et al.,

313    2018). This study shows that differences in levels of *gfp* expression and development of

314    lytic cycle upon induction with mitomycin C in different lysogens, which further

315    demonstrated that the regulation of the same Stx2 phage is dependent on the host strain

316    (this study). RecA interacts with damaged DNA and forms an active form, which causes

317    auto-proteolysis of the prophage repressor (Gimble and Sauer, 1986). Induction of lytic

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318    cycle in Stx prophage results in the production and release of progeny phages and Stx,

319    which is the major pathway to deliver toxin outside of the cells (Neely and Friedman, 1998;

320    Shimizu et al., 2009). Some antibiotics also induce the lytic cycle of Stx prophages in *E.*

321    *coli* O104:H4 and strains of *E. coli* O157:H7 (Bielaszewska et al., 2012; Grif et al., 1998),

322    thus treatment of STEC infections with antibiotics may be more harmful than beneficial.

323    Because both the sequence of the prophage promotors and the host genome differentially

324    impact the induction of Stx prophages (Grif et al., 1998; Zhang et al., 2018), also indicated

325    by this study, conditions of environmental stress that induce Stx prophages are specific to

326    the prophage and its host.

327    **4.3. Induction of Φ11-3088 during sprouting and transduction of non-pathogenic**

328         **strains of *E. coli***

329    Desiccation of microbial cells leads to the accumulation of reactive oxygen species, which

330    oxidizes membrane lipids and DNA (França et al., 2007; Garre et al., 2010). Oxidative

331    stress also induces the RecA-dependent SOS response and expression of Stx prophages

332    including the Stx2 prophage in *E. coli* O104:H4 (Fang et al., 2020, 2017; Łos et al., 2010).

333    Stress associated with drying and rehydration of *E. coli* also induced the λ-prophage (Webb

334    and Dumasia, 1967). Desiccation followed by rehydration of *E. coli* O104:H4 also oxidized

335    membrane lipids and resulted in induction of *recA* and *stx2a*, similarly to our previous

336    study (Fang et al., 2020), indicating that oxidative stress associated with desiccation of

337    seeds prior to germination likely lead to the Stx prophage induction. As a common biocide

338    for seeds decontamination, hypochlorite also induced the cellular oxidation and expression

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339  of *stx2a* (Wang et al., 2020). Overall, sprout production supports Stx prophage induction

340  and transduction.

341  Food matrix and food production were previously shown to allow Stx phage transduction

342  (Imamovic et al., 2009; Nyambe et al., 2017). Solid foods with low fluidity limit the

343  dispersal of phages; thus limiting phage transduction (Imamovic et al., 2009). The

344  concentration of donor and recipient strains that generated transductants in solid foods was

345  above $10^5$ CFU/g (Imamovic et al., 2009). Bean sprouts contain a high microbiological

346  load with the concentration of 5 to 9 log10 CFU/g, and endophytic *Enterobacteriaceae* are

347  among the dominating bacterial groups (Abadias et al., 2008; Martínez-Villaluenga et al.,

348  2008). Sprouting thus provides suitable conditions for phage transduction because drying

349  and dry storage of seeds induces the lytic cycle of Φ11-3088 during rehydration, while

350  rehydration and sprouting bring the phage in contact with potential hosts. In the present

351  study, about one in $10^4$ of the susceptible host population was lysogenized by Φ11-3088;

352  this proportion of transduced cells may be high enough to support transduction in

353  commercial operations, where the cell count of Φ11-3088-carrying *E. coli* is much lower.

354  **4.4. Phage transduction and amplification of load.**

355  Transduction of non-pathogenic strains of *E. coli* amplifies the numbers of STEC

356  contributing to toxin production and thus increases the exposure of humans to Stx, which

357  causes the hemolytic uremic syndrome. The *in vivo* transduction of non-pathogenic,

358  commensal *E. coli* by Stx phages was demonstrated in sheep (Sekse et al., 2008). The

359  present study documents transduction of non-pathogenic *E. coli* with a Stx phage in food

360  prior to consumption, which may further contribute to an increased load of STEC and an

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361  increased exposure to the Stx. Risk related to the presence of Stx2 phages on leafy

362  vegetables and sprouts has not been taken into great consideration, although Stx2 phages

363  frequently occur in commercial salads (Imamovic et al., 2009). During food processing,

364  bacterial stress responses are induced by antimicrobial interventions and food processing

365  using desiccation and low aw, pH and temperatures, which mediates the persistence of

366  viable cells and some stress attributes to the spreading of virulence genes by phages (Abee

367  and Wouters, 1999; Fang et al., 2017). Sprouts production involves rehydration of dry

368  seeds, which allows desiccated STEC to resume their metabolism and to repair cellular

369  damage, but also induces the spread of *stx*-encoding phages via prophage induction. The

370  risk associated with the presence of Stx2 phages during sprouting is primarily dependent

371  on the microbiological condition of the seeds, which are generally not cleaned to eliminate

372  human pathogens (Dechet et al., 2014). Moreover, because *Enterobacteriaceae*, including

373  *E. coli*, are present as endophytes in seeds, external sanitizers cannot eliminate bacterial

374  hazards during germination (Montville and Schaffner, 2004). Effective strategies to control

375  the microbiological condition of seeds before and during sprouting without compromising

376  food quality are urgently needed to reduce microbiological hazards associated with ready-

377  to-eat fresh sprouts (Abadias et al., 2008).

378  **5.  Acknowledgements**

381  **6.  References**

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606    **Figure Legends**

607    **Figure 1.** Cell density of Φ11-3088Δ*stx* lysogenic strains of *E. coli* in the presence of

608    mitomycin C. The optical density was monitored at 600 nm during growth of the following

609    strains: *E. coli* O104:H4Δ*stx2::gfp::amp$^r$* **(Panel A)**, *E. coli* O103:H2 PARC 444 **(Panel**

610    **B)**, *S. sonnei* ATCC 25391 **(Panel C)**, *E. coli* FUA1043 **(Panel D)**, *E. coli* ATCC 31618

611    **(Panel E)**, *E. coli* DH5α **(Panel F)**. Dots in black and grey represent the culture grown in

612    LB and LB supplemented with mitomycin C, representatively. Data shown are three

613    repeats for mitomycin C treatments and one repeat for data of control, except for *E. coli*

614    DH5α.

615    **Figure 2.** Quantification of *gfp* expression in six Φ11-3088Δ*stx*-encoding *E. coli* upon

616    induction by mitomycin C. **(Panel A)**: Exponentially growing cells without induction

617    served as controls. **(Panel B)**: *E. coli* O104:H4Δ*stx2::gfp::amp$^r$* (black), *E. coli* O103:H2

618    PARC444 (dark-grey), *S. sonnei* ATCC 25391 (hatched-white), ETEC ATCC 31618

619    (white), *E. coli* DH5α (light grey), *E. coli* FUA1043 (hatched-grey) were induced by

620    mitomycin C (0.5 mg/L) for 3 h. Expression of GFP and formation of filaments were

621    determined by flow cytometry at a single-cell level. Data are means ± standard deviation

622    for three independent experiments. Different letters denote significant differences among

623    strains of the same sub-population ($P<0.05$).

624    **Figure 3.** Cell counts of *E. coli* O104:H4Δ*stx2::gfp::amp$^r$*, *E. coli* DH5α pJIR750ai, and

625    putative Φ11-3088 lysogens during 96 h of sprouting. *E. coli* on mung bean sprouts were

626    plated on LB agar (white), LB-Amp (dark grey), LB-Chl (light grey), or LB-Amp-Chl

627    (black). Cell counts of chlorine-treated mung beans were below detection limits of 2.3

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628   $\log_{10}$CFU/g. Data are shown as means $\pm$ standard deviation of three independent

629   experiments. Different letters denote significant differences in cell counts among different

630   days (P<0.05).

**Table 1**. Strains of *E. coli* used in this study

| Strain | Virulence factors | Origin | Reference |
|---|---|---|---|
| **pathogenic strains of *E. coli*** | | | |
| O104:H4 11-3088 Δ*stx2::gfp::amp^r* | *agg* | | (Fang et al., 2017) |
| O145:NM 03-6430 | *stx1*, *eae* | Human | |
| O26:H11 05-6544 | *stx1*, *eae* | Human | |
| O45:H2 05-6545 | *stx1*, *eae* | Human | |
| O103:H2 PARC 444 | *pap* | Unknown | (Liu et al., 2015; Mercer et al., 2015 |
| O103:H2 PARC 445* | *pap* | Unknown | |
| O111:NM PARC 447 | *stx1*, *stx2*, *eae* | Unknown | |
| O26:H11 PARC 448 | *eae* | Unknown | |
| O145:NM PARC 449 | *eae* | Unknown | |
| ATCC 31618 | *estIa* | Calf | (Tsen et al., 1998) |
| ECL 13086* | *estIa,estIb,astA*, F4 | Pig | Reference Laboratory for *E. coli* (ECL) of the Université de Montréal |
| ECL 13795* | *estIb*, *astA*, K88 | Pig | |
| ECL13998* | *estIa*, *estIb,astA*, F4 | Pig | |
| ECL 14408* | *estIb*, *astA*, F4 | Pig | |
| ***stx1* and *stx2* negative strains of *E. coli*** | | | |
| FUA1170 | | | |
| FUA1171 | | | |
| FUA1172 | | | |
| FUA1415 | | | |
| FUA1416 | | | |
| FUA1409 | | | |
| FUA1412 | | Human | this study |
| FUA1413 | | | |
| FUA1414 | | | |
| FUA1405* | | | |
| FUA1406* | | | |
| FUA1407* | | | |
| FUA1408* | | | |
| FUA1173* | | | |
| FUA1050 | | | |
| FUA1174 | | | |
| FUA1176 | | Cow vagina | (Wang et al., 2013) |
| FUA1178 | | | |
| FUA1059 | | | |
| FUA1040 | | | |
| FUA1044 | | | |
| FUA1045 | | | |
| FUA1046 | | | |
| FUA1047 | | Cow rectum | This study |
| FUA1048 | | | |
| FUA1049 | | | |
| FUA1042 | | | |
| FUA1043 | | | |

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| Strain | Virulence factors | Origin | Reference |
|---|---|---|---|
| AW1.7 | | | |
| AW1.8 | | | |
| MB10-1 | | | |
| GM9-1 | | | |
| GM9-3 | | Beef processing plant | (Aslam et al., 2004) |
| GM9-5 | | | |
| GM9-8 | | | |
| GM11-2 | | | |
| GM11-3 | | | |
| GM11-4 | | | |
| **Cloning strains of *E. coli*** | | | |
| DH5α, *recA*- | | | Sigma |
| DH5α pJIR750ai | | | |
| **Strains of *Shigella sonnei* and *S. flexneri*** | | | |
| *S. sonnei* ATCC 25391 | | human | ATCC |
| *S. flexneri* A62 | | | this study |

*indicates strains were ampicillin resistant

**Table 2.** Susceptibility of *E. coli* and *Shigella* to lysogenic or lytic infection by Φ11-3088Δ*stx2*.

| Strain | Lysogen | Lysis |
|---|---|---|
| *E. coli* DH5α | + | + |
| *E. coli* O103:H2; PARC 444 | + | - |
| *S. sonnei* ATCC 25391 | + | - |
| *E. coli* ATCC 31618 | + | - |
| *E. coli* FUA 1043 | + | - |
| *E. coli* O103:H2; PARC 445* | | - |
| *E. coli* ECL 14408* | | - |
| *E. coli* ECL 13998* | | - |
| *E. coli* ECL 13795* | | - |
| *E. coli* ECL 13086* | Not determined | - |
| *E. coli* FUA 1405* | | - |
| *E. coli* FUA 1406* | | - |
| *E. coli* FUA 1407* | | - |
| *E. coli* FUA 1408* | | - |
| *E. coli* FUA 1173* | | - |

*indicates strains were ampicillin resistant

**Table 3**. Validation of possible lysogens formed during sprouting for 96 hours. Ampicillin and chloramphenicol resistant isolates were determined for the presence of *gfp* and vector pJIR750ai.

| | no. of *gfp*/pJR750ai positive cells/total no. of cell tested | |
|---|---|---|
| **Time (h)** | ***gfp*** | **pJIR750ai** |
| 24 | 6/28 (21%) | n.d. |
| 48 | 3/27 (11%) | 3/3 |
| 72 | 13/20 (65%) | 8/8 |
| 96 | 12/17 (71%) | 8/8 |

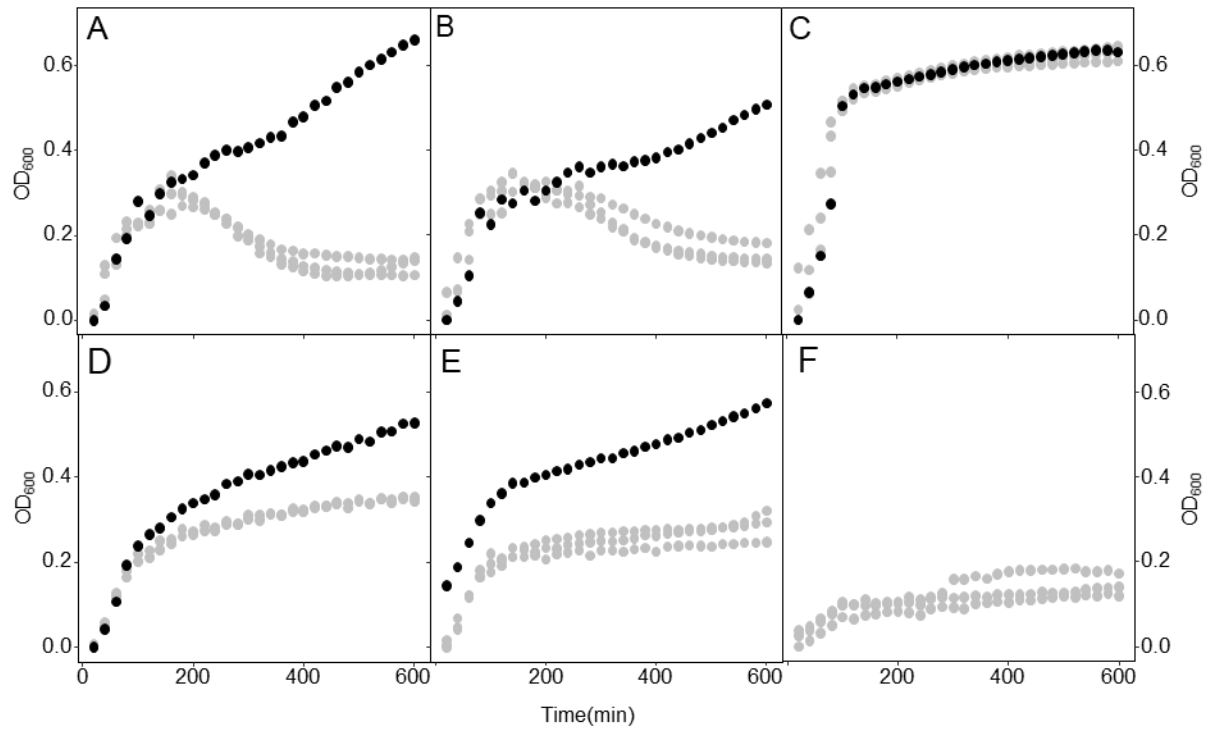n.d. not determined because colonies at 24 h did not re-grow in LB-Amp-Chl.
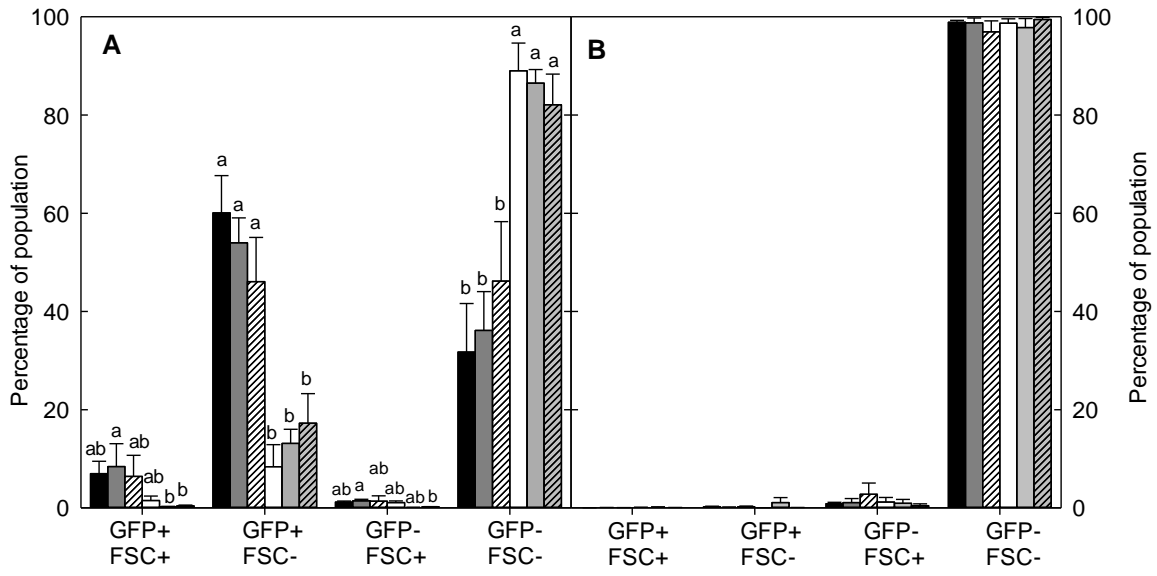
**Figure 1**

**Figure 2**

**Figure 3**