Potential Application of Bacteriophages in Enrichment Culture for Improved Prenatal *Streptococcus agalactiae* Screening

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ABSTRACT

Vertical transmission of *Streptococcus agalactiae* can cause neonatal infections. A culture test in the late stage of pregnancy is used to screen for the presence of maternal *S. agalactiae* for intrapartum antibiotic prophylaxis. For the test, vaginal-rectal swab sampling is immediately followed by enrichment culture and bacterial identification. In some cases, *Enterococcus faecalis* competes with and overgrowths *S. agalactiae* in the enrichment culture. Consequently, the identification test occasionally yields false-negative results. Bacterial viruses, bacteriophages (phages), infect and kill specific host bacteria. In the current study, we explored the feasibility of using phages to minimize the undesirable *E. faecalis* outgrowth and facilitate *S. agalactiae* detection in an experimental setting. Phage mixture was prepared using three phages that specifically infect *E. faecalis*: phiEF24C, phiEF17H, and phiM1EF22. The mixture inhibited the growth of 86.7% (26/30) of *E. faecalis* strains tested in the enrichment broth. When single strains of *E. faecalis* and *S. agalactiae* were inoculated in the enrichment broth containing the phage mixture, bacterial growth was inhibited or facilitated, respectively. Further, several sets of *S. agalactiae* and *E. faecalis* strains were co-cultured, and bacteria were detected on chromogenic agar after the enrichment culture. *S. agalactiae* was dominant after plating a phage mixture-treated

35 co-culture, while it was barely detected after plating the untreated co-culture. Considering
36 these observations, the phage mixture can be employed in the *S. agalactiae* culture test to
37 increase test accuracy.
38
39 Keywords: phage, *Enterococcus faecalis*, *Streptococcus agalactiae*, culture enrichment
40

INTRODUCTION

Streptococcus agalactiae (also called group B streptococcus) is vertically transmitted to the newborn during delivery, and can cause neonatal infections (1, 2). Common early-onset diseases caused by this organism in infants include sepsis and pneumonia, and (rarely) meningitis (1, 2). To prevent such infections, prenatal *S. agalactiae* culture test is recommended in the late stage of pregnancy (1, 2). In the case of a positive test result, the pregnant carrier is prophylactically treated with antibiotics to prevent vertical transmission of *S. agalactiae* during the intrapartum period (1, 2).

For the *S. agalactiae* culture test, the Center of Disease Control and Prevention highly recommends an enrichment culture, followed by conventional *S. agalactiae* identification (3, 4). In the culture test, a swab is taken from the vaginal and anorectal areas, and the samples are inoculated and cultured in an enrichment culture broth selective for *S. agalactiae*. After the enrichment culture, bacterial identification is performed, e.g., by using the Christie-Atkins-Munch-Petersen test, serologic identification, growth on chromogenic agar, and nucleic acid amplification (4). However, although a selective culture broth is used for the enrichment culture, *S. agalactiae* is poorly recovered along with overgrowth of *Enterococcus faecalis* (5-8). This may lead to false-negative results in the subsequent

identification tests (5-8). To address the problem of false-negative results, selective antimicrobial agents to be included in the enrichment broth should be re-evaluated.

Bacteriophages (phages), i.e., bacterial viruses, infect specific bacteria. Some phages infect and lyse bacteria at the specificity level of species and strains. These phage characteristics have been used to eliminate most cells in a bacterial population and facilitate the isolation of less prevalent environmental bacteria that produce novel bioactive compounds (9). Phage applicability for the isolation of food-poisoning microbes in the food microbiology field has also been examined (10). Hence, potentially, phage application might also be used to reduce the unwanted growth of *E. faecalis* and facilitate *S. agalactiae* growth in an *S. agalactiae* enrichment culture in clinical microbiology. Indeed, phages that specifically infect *E. faecalis* have been isolated from environmental samples, such as sewage and canal water (11-13). In the current study, we examined the applicability of *E. faecalis*-specific phages to suppress *E. faecalis* growth in *S. agalactiae* enrichment culture.

MATERIALS AND METHODS

Bacteria, phages, and culture media. Strains of *E. faecalis* (n = 30), *S. agalactiae* (n = 7), *Enterococcus avium* (n = 5), and *Enterococcus faecium* (n = 5) were

75 isolated from vaginal swabs using the Chrom-ID Strepto B test (bioMérieux,
76 Marcy-l'Étoile, France). The swabs were obtained after random sampling at local hospitals
77 in eastern Japan (Table S1). Bacteria were cultured at 37°C under aerobic or microaerobic
78 (i.e., 5% CO_2) condition, as appropriate, based on their specific growth requirements (Table
79 S1).

80 Phage phiEF24C has been isolated and characterized, as described elsewhere (12,
81 14, 15). Phage phiEF17H was newly isolated from canal water in Kochi (Japan). Phage
82 phiM1EF22 was newly isolated from sewage water in Tokyo (Japan) (Table S2). The
83 isolation procedures are described elsewhere (12). *E. faecalis* strains KUEF01, KUEF25,
84 and KUEF27, described in Table S1, were used as host bacteria for phages phiEF24C,
85 phiEF17H, and phiM1EF22, respectively, for phage amplification and plaque assay.
86 Bacterial-phage suspensions were cultured aerobically at 37°C.

87 *Enterococcus* spp. and phages were cultured in tryptic soy broth or agar (TSA),
88 and *S. agalactiae* was cultured in Todd-Hewitt broth (THB), unless stated otherwise.
89 Granada-type broth with slight modification [GBwSM; 25.0 g/l proteose peptone no. 3,
90 14.0 g/l soluble starch, 2.5 g/l glucose, 1.0 g/l pyruvic acid sodium salt, 0.1 g/l cysteine
91 hydrochloride, 0.3 g/l magnesium sulfate, 11.0 g/l 3-(*N*-morpholino)propane sulfonic acid,

10.7 g/l disodium hydrogen phosphate, 0.5 mg/l crystal violet, 10 mg/l colistin sulfate, 10 mg/l metronidazole, and 15 mg/l nalidixic acid, pH 7.4] was originally prepared as the *S. agalactiae* enrichment broth (16, 17). Alternatively, the pigmented enrichment Lim broth (modified Lim broth; Kyokuto Pharmaceutical Industrial, Tokyo, Japan) was used as an *S. agalactiae* enrichment broth. Unless stated otherwise, all culture media were purchased from Becton, Dickinson, and Co. (Franklin Lakes, NJ). All chemicals and reagents were purchased from Nacalai Tesque (Kyoto, Japan) and FUJIFILM Wako Pure Chemical (Osaka, Japan).

Phage genome sequencing. After phage amplification, phage particles were purified from 500 ml of phage lysate by CsCl density-gradient centrifugation, as described elsewhere (18). Phage genomic DNA was then prepared by phenol-chloroform extraction of the collected purified phage band, as described (18). Shotgun library was prepared for each phage DNA using the GS FLX Titanium rapid library preparation kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instruction. The libraries were analyzed using a GS Junior 454 sequencer (Roche Diagnostics). The sequence reads were assembled using the 454 Newbler software (version 3.0; 454 Life Sciences, Branford, CT). The genome sequences were analyzed by using BLASTn at the National Center for

109 Biotechnology Information (NCBI;
110 https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&
111 LINK_LOC=blasthome; last accessed: 5 May, 2018). The genomes were annotated using a
112 prokaryotic genome annotation pipeline, DFAST (https://dfast.nig.ac.jp/) (19, 20).

113 **Multi-locus sequence typing (MLST) of *E. faecalis* strains.** *E. faecalis* strains
114 were cultured overnight, bacterial DNA was extracted, and MLST analysis was performed,
115 according to the procedures described elsewhere (21). The sequence alleles were analyzed
116 using the *E. faecalis* MLST database (https://pubmlst.org/efaecalis/; last accessed: 5
117 January, 2018) to designate sequence types (STs) (22). The concatenating allele sequences
118 were analyzed using MEGA 7.0.18, and sequence alignment implemented in ClustalW was
119 followed by phylogenetic tree construction by the UPGMA method (23).

120 **Examination of antibacterial activity of *E. faecalis* to *S. agalactiae*.** The anti-*S.*
121 *agalactiae* activity of *E. faecalis* was examined by spot-on-lawn assay, as described
122 elsewhere (24). Briefly, 200 μl of overnight bacterial culture of a single *S. agalactiae* strain
123 were mixed with a melted 0.5% (w/v) soft agar and plated onto 1.5% (w/v) agar. One
124 microliter of *E. faecalis* overnight culture was spotted on the solidified top agar. After
125 incubation overnight at 37°C in a microaerophilic condition, *S. agalactiae* growth around

126 the spotted *E. faecalis* was examined.

127 **Analysis of phage lytic activity.** The phage host range was determined by a streak
128 test, as described elsewhere (12, 15). Briefly, 200 μl of overnight bacterial culture of a
129 single bacterial strain were mixed with a melted 0.5% (w/v) soft agar and plated onto 1.5%
130 (w/v) agar. Phage suspension (ca. $1.0 \times 10^{8-9}$ PFU/ml) was streaked onto the solidified top
131 agar. After incubation overnight at 37°C, bacterial lysis, with or without plaque formation,
132 was examined.

133 **Analysis of bacterial growth inhibition by the phage mixture in *S. agalactiae***
134 **enrichment broths.** Bacteria were cultured until optical density of 0.4–0.6 at 600 nm. After
135 washing and diluting with GBwSM, $3.0–5.0 \times 10^8$ CFU/ml bacterial suspension was
136 prepared in GBwSM. Each phage suspension was diluted in THB to ca. 3.0×10^8 PFU/ml,
137 3.0×10^6 PFU/ml, and 3.0×10^4 PFU/ml. Phage mixtures were prepared by mixing equal
138 volumes of phage suspensions at the same dilution. Then, 5 μl of bacterial suspension and 5
139 μl of phage mixture were added to 140 μl of GBwSM in a well of a flat-bottomed
140 polystyrene 96-well plate (AS ONE Co., Osaka, Japan). In the experiments, 5 μl of THB
141 was used instead of the bacterial suspension and/or phage mixture as a control. The 96-well
142 plate was incubated at 37°C and sample turbidity was measured over time at 595 nm, using

143 a Multiskan JX spectrophotometer (Thermo Labsystems, Stockholm, Sweden). The
144 experiments were conducted in triplicate, and the growth curves were then plotted using
145 averaged values with standard deviations.

146 **Analysis of bacterial densities in *S. agalactiae* and *E. faecalis* co-culture with**
147 **phage mixtures.** Rifampicin-resistant mutant clone of *S. agalactiae* was isolated by
148 aerobically culturing *S. agalactiae* strain KUGBS2 on TSA containing 20 μg/ml rifampicin
149 at 37°C for 2 d. The putative mutant clones were re-purified at least three times; each
150 re-purification round was repeated for 1 d under the same incubation conditions. One
151 resultant rifampicin-resistant mutant clone of strain KUGBS2 was obtained and was
152 tentatively designated as strain KUGBS2rif. *S. agalactiae* strain KUGBS2rif and *E.*
153 *faecalis* strain KUEF08 were cultured individually until optical density of 0.4–0.6 at 600
154 nm. After diluting with the enrichment broth, suspensions of 3.0×10^4 CFU/ml *S.*
155 *agalactiae* strain KUGBS2rif and 3.0×10^7 CFU/ml *E. faecalis* strain KUEF08 were
156 prepared. Each phage suspension was diluted with THB to ca. 3.0×10^6 PFU/ml or $3.0 \times$
157 10^4 PFU/ml. By mixing equal volumes of phage suspensions at the same dilution, mixtures
158 of two different dilutions of phages were prepared.

159 For the experiment, 100 μl each of *S. agalactiae* strain KUGBS2rif and *E. faecalis*

160 strain KUEF08, and 300 μl of phage mixture were added to 10 ml of the *S. agalactiae*
161 enrichment broth. As negative controls, the same volume of THB was added instead of
162 bacterial suspensions and/or phage suspensions. The mixtures were microaerobically
163 incubated at 37°C for 24 h. Total bacterial density, and *S. agalactiae* strain KUGBS2rif and
164 *E. faecalis* strain KUEF08 densities were determined. Total bacterial densities were
165 determined on TSA. TSA supplemented with 20 μg/ml rifampicin and
166 *Enterococcus*-selective agar (EF agar base "Nissui"; Nissui Pharmaceutical Co., Tokyo,
167 Japan) were used to determine the densities of *S. agalactiae* strain KUGBS2rif and *E.*
168 *faecalis* strain KUEF08, respectively. *S. agalactiae* strain KUGBS2rif did not grow on the
169 *Enterococcus*-selective agar; conversely, *E. faecalis* strain KUEF08 did not grow on TSA
170 containing 20 μg/ml rifampicin.

171 **Detection of bacteria on chromogenic selective agar after *S. agalactiae* and *E.***
172 ***faecalis* co-culture with phage mixtures.** *S. agalactiae* and *E. faecalis* were cultured
173 individually until optical density of 0.4–0.6 at 600 nm. *S. agalactiae* and *E. faecalis*
174 cultures were diluted with THB to ca. 3.0–5.0 × 10^4 CFU/ml and ca. 3.0 × 10^7 CFU/ml,
175 respectively. After dilution of individual phage suspensions in THB to ca. 1.0 × 10^7 PFU/ml,
176 phage mixture was prepared by mixing equal volumes of the diluted phage suspensions.

177 For the experiment, 30 μl each of bacterial suspensions of *S. agalactiae* and *E.*
178 *faecalis*, and 30 μl of phage mixture were added to 3 ml of the enrichment broth. As a
179 negative control, the same volume of THB was added instead of the phage mixture. After
180 24-h incubation at 37°C, a loop-full of the suspension was inoculated on the Chrom-ID
181 Strepto B agar (bioMérieux). After 24-h incubation at 37°C in darkness, colony color and
182 appearance on agar plates were examined. All incubations were made under
183 microaerophilic conditions.

184 **Accession numbers.** The phiEF17H and phiM1EF22 genome sequences were
185 deposited in the GenBank under the accession numbers AP018714 and AP018715,
186 respectively.

187 **Statistical analysis.** The data were statistically analyzed using EZR (Saitama
188 Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user
189 interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (25).
190 Student's *t*-tests were used to analyze differences between bacterial densities in different
191 treatments. The value of $P < 0.01$ was considered to indicate statistically significant
192 difference.

193 **Data availability.** By publishing in the journal, the authors agree that, subject to

194 requirements or limitations imposed by local and/or U.S. Government laws and regulations,
195 any materials and data that are reasonably requested by others are available from a publicly
196 accessible collection or will be made available in a timely fashion, at reasonable cost, and
197 in limited quantities to members of the scientific community for noncommercial purposes.
198 The authors guarantee that they have the authority to comply with this policy either directly
199 or by means of material transfer agreements through the owner.
200

201 **RESULTS AND DISCUSSION**

202 **Phage characteristics.** Phages phiEF24C, phiEF17H, and phiM1EF22 were used
203 in the current study. Phage phiEF24C, one of the well-studied *Enterococcus* phages, is
204 classified into the family *Myoviridae* subfamily *Spounavirinae* (26). Based on the
205 whole-genome sequence similarities to the phage phiEF24C genome, phages phiEF17H
206 and phiM1EF22 share viral taxonomy with that phage (Table S3). Phages sharing this
207 particular viral taxonomy are highly virulent toward host bacteria (27).

208 MLST analysis of the *E. faecalis* vaginal swab isolates (Fig. 1) revealed that
209 43.3% (13/30) of *E. faecalis* strains were phylogenetically closely related, representing
210 either ST16 or ST179. The remaining strains [56.7% (17/30)] were genetically diverse.

211 Moreover, *E. faecalis* strains, which interfere *S. agalactiae* culture tests, do not seem to
212 show antibacterial activity (e.g., bacteriocin production) to the *S. agalactiae*, while some of
213 them are able to show anti-bacterial activity to a variety of bacteria (5, 28). Testing the
214 antibacterial activity to *S. agalactiae* strains by the spot-on-lawn assay, no anti-*S.*
215 *agalactiae* activity was observed among these *E. faecalis* strains.

216 Phage lytic activity was examined by a streak test using these *E. faecalis* strains
217 (Fig. 1). Phages phiEF24C, phiEF17H, and phiM1EF22 showed lytic activity toward
218 63.3% (19/30), 76.7% (23/30), and 66.7% (20/30), respectively, of the tested *E. faecalis*
219 strains. Lytic activities of the three phages with other bacterial vaginal swab isolates (*E.*
220 *avium*, *E. faecium*, and *S. agalactiae* strains) were also examined, but no lytic activity was
221 observed.

222 **Lytic activity of the phage mixture as an *E. faecalis*-selective antimicrobial**
223 **agent.** Phages phiEF24C, phiEF17H, and phiM1EF22 lysed different *E. faecalis* strains and
224 also some common strains. Theoretically, a combination of these three phages lysed a
225 broader range of *E. faecalis* strains than any single phage tested. Phage mixture containing
226 the three phages was prepared by mixing phage particles in 1:1:1 ratio. The lytic spectrum
227 of the phage mixture was then examined by using a streak test. The phage mixture showed

228 lytic activity toward 86.7% (26/30) of *E. faecalis* strains tested (Fig. 1). Four *E. faecalis*
229 strains were not lysed by the phages, namely, KUEF02 (MLST ST47), KUEF18 (ST64),
230 KUEF15 (ST30), and KUEF28 (ST179). The phage mixture did not show any lytic activity
231 with the other tested bacteria, i.e., *E. avium*, *E. faecium*, and *S. agalactiae*.

232 To evaluate the effect of the phage mixture on the growth of *E. faecalis* in the
233 enrichment broth, *E. faecalis* growth was monitored in the presence of three dilutions of the
234 phage mixture in GBwSM. The medium lacked the customary pigment enhancer
235 methotrexate to improve data reliability (16, 17). After bacterial inoculation, phage mixture
236 was added to the multiplicity of infection (MOI) of each phage of 1, 10^{-2}, or 10^{-4}. For the *S.*
237 *agalactiae* enrichment culture, 18–24-h incubation is recommended (3, 4). Consequently,
238 the turbidity of cultures of 30 *E. faecalis* strains described in Fig. 1 was recorded in the
239 presence or absence of the phage mixture over 24 h.

240 To simplify data interpretation, *E. faecalis* growth curves were tentatively
241 categorized into four patterns (A–D), after comparing with the growth curve of untreated *E.*
242 *faecalis* (Fig. 2A). For strains representing pattern A growth curve, *E. faecalis* growth was
243 inhibited throughout the experiment. For strains representing pattern B growth curve, *E.*
244 *faecalis* growth was initially inhibited (8 h post inoculation) and then gradually recovered.

For strains representing pattern C growth curve, bacteria showed an initial growth but were then lysed. Pattern C was a typical phage lysis pattern at the lower MOI tested. *E. faecalis* strains that exhibited growth patterns A–C were inhibited by the phage treatment. The tested *E. faecalis* strains treated with higher concentrations of phage mixtures and ones that were most sensitive to phage mixture treatments tended to exhibit growth patterns A, B, and C, in the order of strongest to weakest inhibition. On the other hand, phage treatment did not inhibit *E. faecalis* strains exhibiting growth pattern D. The four phage-insensitive *E. faecalis* strains (KUEF02, KUEF18, KUEF15, and KUEF28), and *E. avium*, *E. faecium*, and *S. agalactiae* strains exhibited growth pattern D. The reasons for such tentative growth pattern categorization (i.e., growth patterns A-D) were probably due to (1) different sensitivity of *E. faecalis* strains to phages, and (2) the use of three dilutions of the phage mixture in experiments.

The tentative grouping of growth patterns of the 30 tested *E. faecalis* is summarized in Fig. 2B. Phage mixture treatments at the highest and the second highest phage density (i.e., MOI 1 and 10^{-2}, respectively) inhibited the growth of 86.7% (26/30) of *E. faecalis* strains, which was in agreement with the results of the streak test (Fig. 1). On the other hand, when the phage density was reduced (i.e., MOI 10^{-4}), the number of *E.*

faecalis strains whose growth was inhibited was reduced (76.7%, 23/30 strains). The latter 23 strains largely represented growth pattern C, probably because the input phage titer was much lower than that in the other phage mixture treatments. The seven remaining *E. faecalis* strains that exhibited growth pattern D included the four phage mixture-insensitive strains mentioned above and strains KUEF03 (ST3), KUEF19 (ST16), and KUEF30 (ST16). In the current study, a correlation between growth patterns and STs was not observed among the *E. faecalis* strains tested.

Based on these observations, we concluded that the phage mixture inhibited the growth of 86.7% (26/30) of *E. faecalis* strains tested, when used at sufficiently high density (i.e., MOI of at least 10^{-2}).

The effect of phage mixture on *S. agalactiae* and *E. faecalis* cell densities in experimental enrichment cultures. Phage mixture may have been contaminated with this anti-*S. agalactiae* agents during phage mixture preparation (i.e., during phage propagation on the *E. faecalis* host). However, incubation of phage mixture with *S. agalactiae* did not significantly affect bacterial viability, compared with a THB-treated negative control (Fig. S1), excluding the possibility of phage mixture contamination with anti-*S. agalactiae* substances.

279 The effects of phage mixture were then examined in co-cultures of *E. faecalis* and
280 *S. agalactiae* in the Granada-type enrichment broth. The rifampicin-resistant mutant clone
281 of strain KUGBS2, KUGBS2rif, was isolated. *S. agalactiae* strain KUGBS2rif and *E.*
282 *faecalis* strain KUEF08 were used to quantify viable bacterial individually. To mimic the
283 situation that *S. agalctaie* was poorly recovered, *S. agalactiae* strain KUGBS2rif and *E.*
284 *faecalis* strain KUEF08 were inoculated into GBwSM at 3.0×10^2 CFU/ml and 3.0×10^5
285 CFU/ml, respectively. Either of the two dilutions of phage mixture (at MOI of each phage
286 of 10^{-1} and 10^{-3} to *E. faecalis*) were added. As a negative control, THB was used instead of
287 the phage mixture.

288 Changes in bacterial cell density (total bacteria, *S. agalactiae*, and *E. faecalis*)
289 were then monitored over time (Fig. 3). Based on the determined total bacteria numbers,
290 bacteria grew exponentially for up to 12 h, following which the cultures entered the
291 stationary phase of growth. Hence, 12–24-h incubation was sufficient to achieve bacterial
292 enrichment in that particular experimental setting. Moreover, changes in *E. faecalis* and *S.*
293 *agalactiae* cell densities were then evaluated. In the negative control group (i.e., no phage
294 treatment), *E. faecalis* grew much better than *S. agalactiae*. In the phage treatment groups,
295 the opposite was observed. After 12 and 24 h, in the phage treatment groups, cell density of

296 *E. faecalis* was significantly lower than that of *S. agalactiae* ($P < 0.01$).

297 Several types of enrichment broths are commercially available for *S. agalactiae*
298 culture test. In addition to the GBwSM medium, we also evaluated the effectiveness of
299 phage treatment of an *E. faecalis* and *S. agalactiae* co-culture in the commercially available
300 pigmented enrichment Lim broth (Fig. S2). The experiments were performed as described
301 above. Inhibition of *E. faecalis* growth, compared with the untreated group, was observed
302 in phage treatment groups at both MOIs of each phage tested (i.e., 1 and 10^{-2}) (Fig. S3).
303 This indicated that phage mixture inhibited *E. faecalis* growth and facilitated *S. agalactiae*
304 growth in both *S. agalactiae* enrichment broths.

305 **Efficient detection of *S. agalactiae* after enrichment culturing in the presence**
306 **of phage mixture.** In the *S. agalactiae* culture test, bacteria are generally identified in a
307 culture aliquot after enrichment culture. Consequently, we then evaluated the efficiency of
308 *S. agalactiae* identification after the experimental enrichment culture. As the identification
309 assay, we used growth on the *S. agalactiae* chromogenic agar, in which *S. agalactiae*
310 colonies are distinguished from *E. faecalis* colonies based on color. We tested several *S.*
311 *agalactiae–E. faecalis* combinations. *E. faecalis* and *S. agalactiae* strains were first
312 inoculated at 100:1 ratio in the Granada-type enrichment broth, the phage mixture was

313 added (at an MOI of each phage at 10^{-1} to *E. faecalis*), and the cultures incubated.
314 Enrichment culture aliquots were plated on chromogenic agar, and the resultant colony
315 appearance evaluated (Fig. 4). After enrichment of all phage-treated *S. agalactiae–E.*
316 *faecalis* sets, *S. agalactiae* colonies were dominant on the agar plates. By contrast, in
317 enrichment cultures without phage treatment, only few *S. agalactiae* colonies were
318 observed on the chromogenic agar, while *E. faecalis* colonies were dominant. The same
319 experiment was performed using the modified Lim broth and several *S. agalactiae–E.*
320 *faecalis* combinations (Fig. S4). The data were in agreement with observations made using
321 the GBwSM medium. Thus, the phage mixture treatment improved the *S. agalactiae*
322 culture test by inhibiting the undesirable growth of *E. faecalis*, even when the initial *E.*
323 *faecalis* cell density was as high 10^5 CFU/ml.

324 **Phage application potential in the clinical setting.** In the current study, we
325 showed that a specific phage mixture effectively inhibited the growth of *E. faecalis* in an *S.*
326 *agalactiae* culture test in the experimental setting. Before reagent manufacture and clinical
327 application, phage composition in the phage mixture, the usage per assay (i.e., volume and
328 phage density), storage of the phage mixture, and production cost should be optimized.

329 As a first consideration, strains insensitive to the phage mixture may occur at a

330 higher than expected rate in the clinical setting. To address this issue, phage sensitivity of *E.*
331 *faecalis* strain should be constantly examined. The effective spectrum of phages in a
332 mixture can be modified by replacing and/or adding other phages, including newly-isolated
333 phages, naturally-evolved phages, and/or genetically-modified phages (29, 30). E.g., by
334 adding six newly isolated phages to the phage mixture developed in the current study, we
335 increased the inhibition efficiency among the tested *E. faecalis* strains to 96.7% (29/30)
336 (data not shown). Hence, updating the composition of the phage mixture will help to
337 address the problem associated with the phage-insensitive strains.

338 Next, we considered the usage per assay. Assuming that the volume of the
339 enrichment broth is ca. 3–4 ml, a drop of phage mixture suspension (i.e., 30–50 μl of a
340 solution of ca. 1.0×10^7 PFU/ml of each phage) would suffice for an assay. Such usage per
341 assay is in line with the usage of phage mixture in experiments performed in the current
342 study (Fig. 4). Thus, phage mixture complies with the usage expected in a clinical setting.

343 Moreover, the quality of the phage mixture should be guaranteed to allow its
344 commercialization. Phages are generally stable at 4°C in culture media for a certain period
345 of time (31, 32), which suggests that phage products can be distributed on a market-scale
346 using the cold chain. Accordingly, we examined the infectious density of each phage

347 solution during storage at 4°C for 270 d, and we did not observe any substantial reduction
348 in phage particle density during this time period (Fig. S5). Shelf life of culture medium is
349 generally up to 6 months (33), and no loss in sensitivity in *S. agalactiae* enrichment broths
350 were observed after at least 4 months (34). Thus, the stability of the phage mixture
351 appeared to be in line with the storage of the enrichment broths.

352 Finally, the use of phage mixture should be cost-effective. Phage production on a
353 bioreactor-scale has been recently investigated because of increased interest in phage
354 therapy. Consequently, the cost of phage production is estimated to be $\$4.4 \times 10^{-13}$/phage
355 particle (35-37). Hence, the calculated cost of phage mixture per assay in the current study
356 is only $\$2.2 \times 10^{-7}$ ($\$4.4 \times 10^{-13}$/phage particle multiplied by the number of phage particles
357 in the mixture, i.e., 1.0×10^{7} PFU/ml $\times$ 50 μl). Several commercial phage companies have
358 been founded worldwide (38). Thus, phage mixture may, in theory, be produced for the *S.*
359 *agalactiae* culture test in a cost-effective manner.

360 After commercialization of the phage mixture, special attention should be devoted
361 to the biological characteristics of phages to avoid laboratory accidents, since phage
362 products are not commonly used in the clinical microbiology. Phages multiply
363 exponentially and exceed bacterial growth, which might lead to phage contamination and

364 interference with *E. faecalis* culture tests in a clinical laboratory. To avoid such accidents,
365 appropriate safety precautions should be instated when preparing to use phage products.
366 These can include change of gloves after usage, aseptic handling at a specified bench, and
367 storage in a specific cabinet (39). We believe that that the phage mixture satisfies the usage
368 in terms of application volume and density, as long as the knowledge and safety precautions
369 for phage products are disseminated and implemented.

370 Considering the above, we anticipate that the phage mixture will become
371 commercially available for *S. agalactiae* enrichment broths in the future and will be used in
372 the clinical setting.

373

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379 There are no conflicts of interest to declare.

380

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486

487

488 **Figure legends**

489

490 **FIG 1** *E. faecalis* strains isolated from vaginal swabs and their sensitivity to phages.
491 Phylogenetic tree of *E. faecalis* strains was constructed based on the concatenated MLST
492 alleles. In the phylogenetic tree, *E. faecalis* strain names are followed by STs in brackets.
493 Phage sensitivities to each phage and phage mixture are shown below the phylogenetic tree.

494

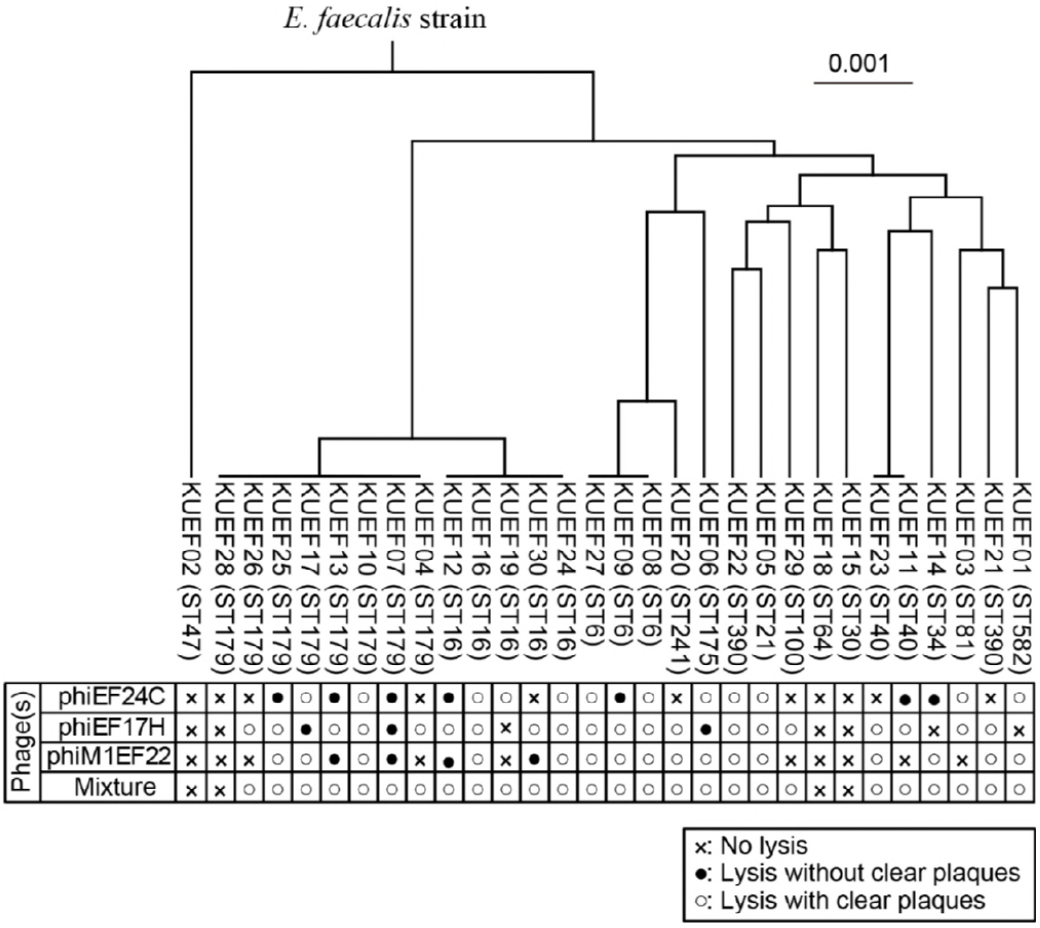
495 **FIG 2** Effects of phage mixture treatment on *E. faecalis* growth in GBwSM. (A) Four
496 growth patterns of *E. faecalis* strains treated with phage mixture. *E. faecalis* strains were
497 treated, or not, with the phage mixture, and culture turbidities were evaluated by measuring
498 optical density at 595 nm (OD595) over time. Thirty *E. faecalis* strains isolated from
499 vaginal swabs, described in Fig. 1, were individually tested. The growth curves were
500 graphed using means and standard deviations from triplicate experiments. The growth
501 curves of phage-treated *E. faecalis* strains were categorized into four types, patterns A–D,
502 after comparing with the growth curves of untreated *E. faecalis* strains. For the growth
503 patterns A–C, phage treatments with all tested phage dilutions inhibited *E. faecalis* growth
504 compared with the negative control (i.e., no phage treatment). In growth pattern D, phage

505 mixture did not inhibit bacterial growth. The representative growth curves were presented:
506 pattern A, strain EF08 treated with phage mixture at MOI of each phage to *E. faecalis* at 1;
507 pattern B, strain EF22 treated with phage mixture at MOI of each phage to *E. faecalis* at
508 10^{-2}; pattern C, strain EF05 treated with phage mixture at MOI of each phage to *E. faecalis*
509 at 10^{-4}; pattern D, strain EF02 treated with phage mixture at MOI of each phage to *E.*
510 *faecalis* at 1. (B) Summary of the effect of phage mixture treatments on *E. faecalis* growth
511 when three different phage mixture dilutions were tested. Phage densities (MOI) for each
512 phage type were 1, 10^{-2}, or 10^{-4}. *E. faecalis* growth patterns were classified based on the *E.*
513 *faecalis* growth patterns described in Fig. 2A, and are summarized as cumulative bar
514 graphs.
515
516 **FIG 3** Growth of *E. faecalis* and *S. agalactiae* co-cultures in the presence or absence of
517 phage mixtures in GBwSM. No phage treatment (A); or treatment with phages at 10^{-3} at (B)
518 or 10^{-1} (C) MOI of each phage to *E. faecalis*. The means with standard deviations were
519 calculated from triplicate experiments, and are plotted as points with error bars. Time points
520 at which *S. agalactiae* density was significantly higher than that of *E. faecalis* are indicated
521 by asterisks ($P < 0.01$; Student's *t*-test).

522

523 **FIG 4** Bacterial identification on chromogenic agar after experimental enrichment
524 co-culture of *S. agalactiae* and *E. faecalis*. Combinations of single strains of *E. faecalis* and
525 *S. agalactiae* were used to inoculate GBwSM, and were cultured in the presence of the
526 phage mixture (MOI of each phage to *E. faecalis*: 10^{-1}) or THB. After enrichment culture,
527 aliquots were spread on the chromogenic agar, and the resultant bacterial colonies were
528 evaluated. Colonies of *S. agalactiae* and *E. faecalis* are red and blue, respectively. Left and
529 right panels, photographs of chromogenic agar plates inoculated with enriched cultures
530 treated with THB or phage mixtures, respectively. Representative data for three out of five
531 *S. agalactiae–E. faecalis* sets are shown, namely, KUGBS2–KUEF08 (A), KUGBS1–
532 KUEF24 (B), and KUGBS6–KUEF26 (C).

533



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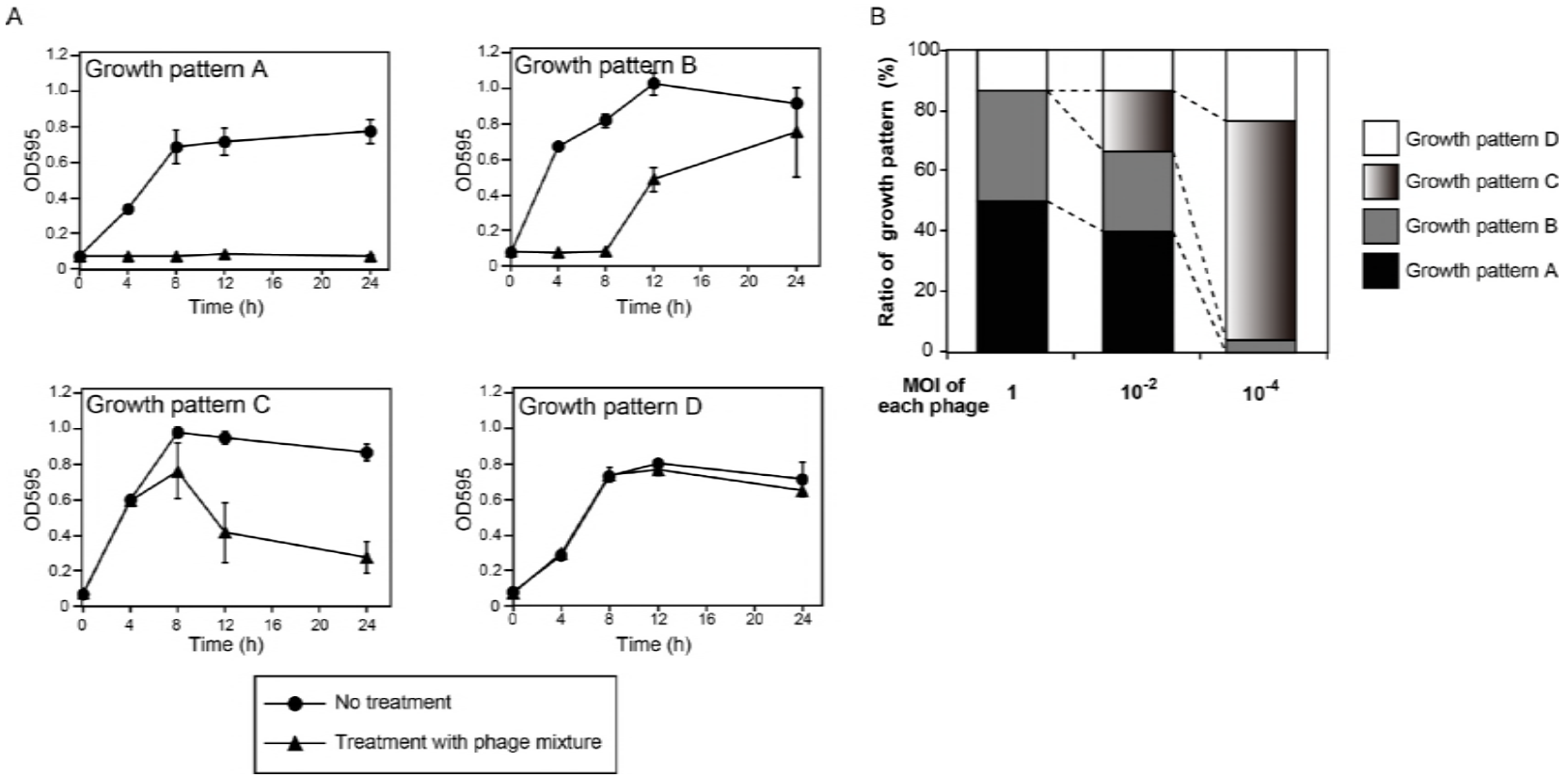

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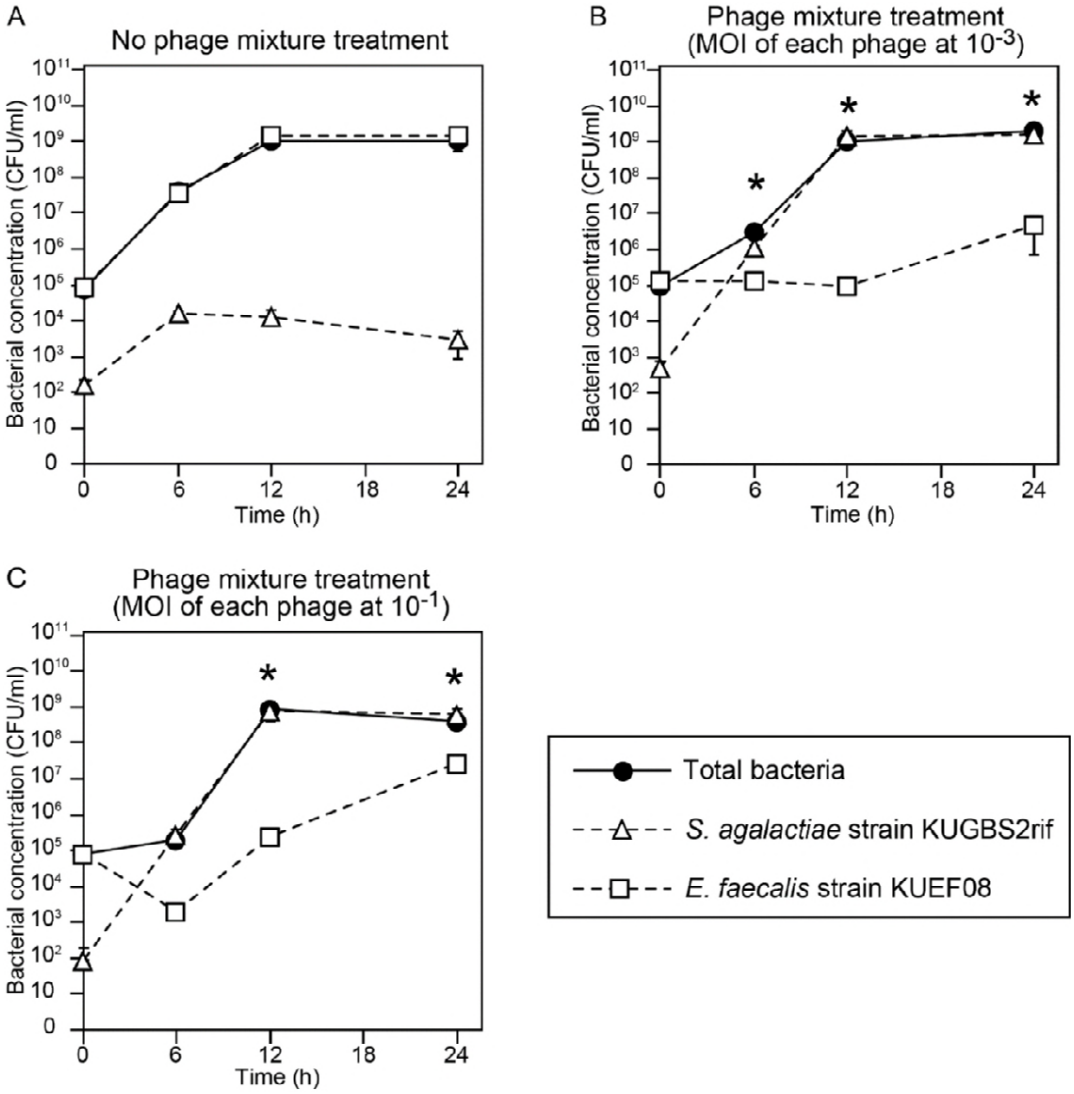

FIG 3 Growth of *E. faecalis* and *S. agalactiae* co-cultures in the presence or absence of phage mixtures in GBwSM. No phage treatment (A); or treatment with phages at 10^{-3} at (B) or 10^{-1} (C) MOI of each phage to *E. faecalis*. The means with standard deviations were calculated from triplicate experiments, and are plotted as points with error bars. Time points at which *S. agalactiae* density was significantly higher than that of *E. faecalis* are indicated by asterisks ($P < 0.01$; Student's *t*-test).

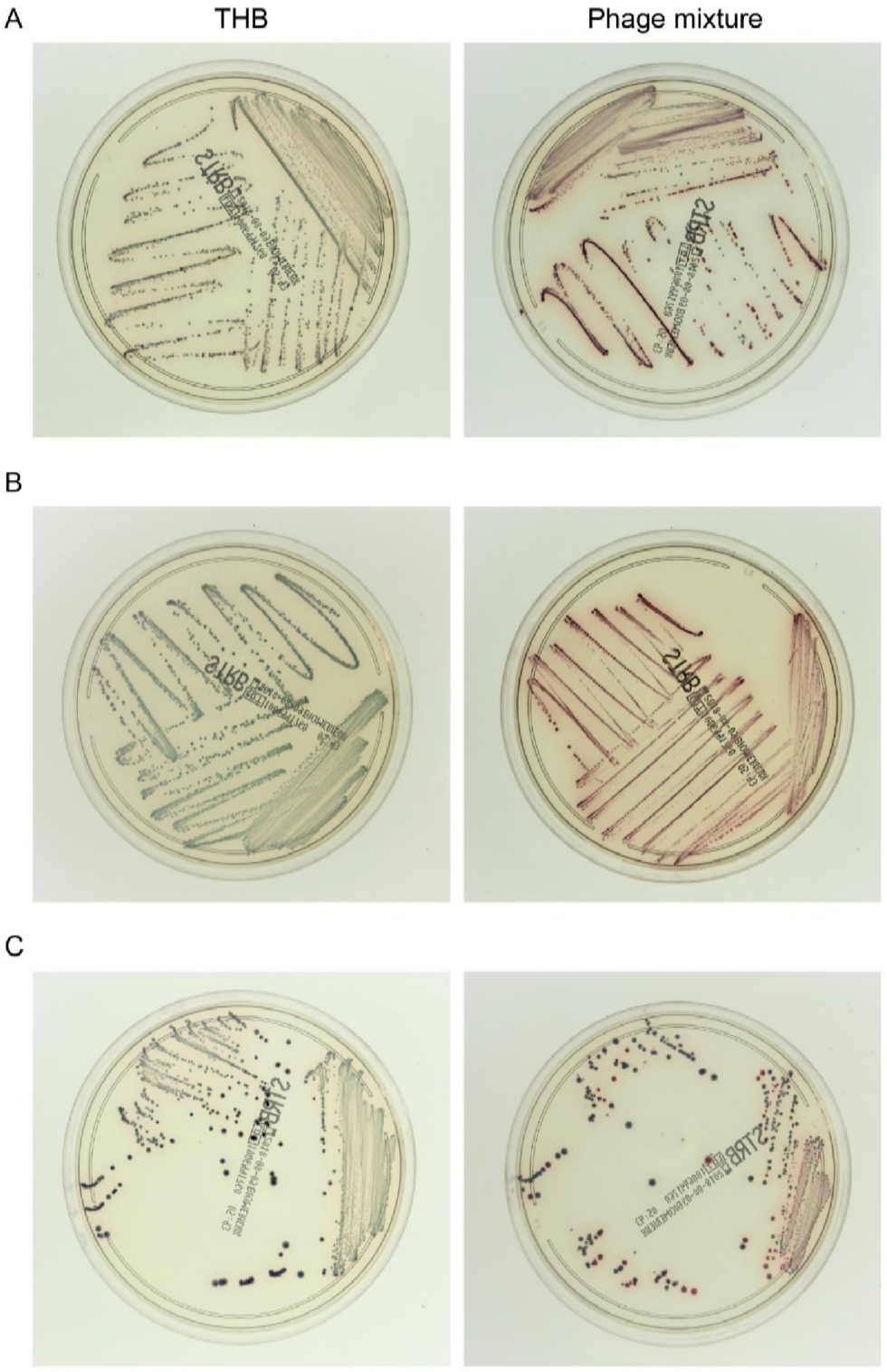

FIG 4 Bacterial identification on chromogenic agar after experimental enrichment co-culture of *S. agalactiae* and *E. faecalis*. Combinations of single strains of *E. faecalis* and *S. agalactiae* were used to inoculate GBwSM, and were cultured in the presence of the phage mixture (MOI of each phage to *E. faecalis*: 10^{-1}) or THB. After enrichment culture, aliquots were spread on the chromogenic agar, and the resultant bacterial colonies were evaluated. Colonies of *S. agalactiae* and *E. faecalis* are red and blue, respectively. Left and right panels, photographs of chromogenic agar plates inoculated with enriched cultures treated with THB or phage mixtures, respectively. Representative data for three out of five *S. agalactiae–E. faecalis* sets are shown, namely, KUGBS2–KUEF08 (A), KUGBS1–KUEF24 (B), and KUGBS6–KUEF26 (C).