



# Effects of heterologous genome microinjection on the enlargement of *Enterococcus faecalis* protoplasts

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## ABSTRACT

The lactic acid bacterium *Enterococcus faecalis* genomic DNA and seven phylogenetically distant bacterial genomic DNAs were microinjected into 126 enlarged protoplasts of *E. faecalis*. After the microinjection, a time-lapse observation was performed on how the cells enlarged. Most cells did not stop enlarging. The enlargement patterns were compared with the enlargement of *E. faecalis* protoplasts not treated by microinjection (control). They were clustered into three groups, with different levels and speeds of protoplast enlargement. The statistical analyses showed that the protoplasts injected by *E. faecalis* and four of the seven phylogenetically different bacterial genomic DNAs had enlargement patterns significantly different from those of the control. Thus, injected genomic DNAs affected the protoplast enlargement. Most of the affected cells, including the *E. faecalis* genome, had weakened enlargement.

## 1. Introduction

The horizontal transfer of genetic elements greatly influences on bacterial evolution (Garcia-Vallvé et al., 2000; Koonin et al., 2001; Nishida and Oshima, 2019). However, the mechanism of the horizontal transfer is uncertain, except for plasmids and viruses. Furthermore, to our knowledge, any horizontal transfers of bacterial chromosomes have not been detected and reported. Thus, it has been uncertain whether bacterial cells can control the genetic information when incorporating heterologous chromosomes.

Horizontally transferred plasmids and viruses have lower guanine-cytosine (GC) content than their host chromosomes (Nishida, 2012; Rocha and Danchin, 2002). However, it is uncertain whether such a phenomenon also occurs in horizontally transferred chromosomes.

The present genome manipulation technology is based on the repetition of existing gene manipulation (Itaya et al., 2005; Lartigue et al., 2007). The host bacteria cells used in genome manipulation, such as *Bacillus subtilis* and *Mycoplasma mycoides*, are limited (Itaya et al., 2005; Lartigue et al., 2007). In addition, such an experiment is time-consuming, labor-intensive, and costly.

Thus, we focused on microinjection technology. Unfortunately, normal bacterial cells are too small to microinject. Instead, bacterial protoplasts are generated by the cell wall lyses, which can enlarge without cell division under suitable culture conditions where cell wall

synthesis is inhibited (Kami et al., 2019; Kuroda et al., 1998; Kusaka, 1967; Lederberg, 1956; Nakamura et al., 2011; Nishino et al., 2018; Takahashi et al., 2016). We succeeded in enlarging the cells of the lactic acid bacterium *Enterococcus faecalis* and in microinjecting the fluorescent proteins into their enlarged cells (Takahashi et al., 2020).

The *E. faecalis* protoplast enlargement requires DNA replication (Kami et al., 2019; Tsuchikado et al., 2020). Thus, when DNA replication stops, cell enlargement also stops. There are two possible causes. One is that the replicated genomic DNA is directly involved in the plasma membrane enlargement. It has been reported that the DNA is bound to the plasma membrane of bacteria (Earhart et al., 1968; Leibowitz and Schaechter, 1975; Marvin, 1968; Sueoka and Quinn, 1968). Therefore, if the attachment is required only for cell division, it may not be essential for protoplast enlargement. The other reason is that the replication and plasma membrane synthesis systems are linked; i.e., both stand side by side and do not function independently. Thus, in the former case, the injection of the *E. faecalis* genomic DNAs may promote plasma membrane synthesis. In the latter case, the effects of the genomic DNA injection on the replication system may affect the cell enlargement.

This study microinjected the *E. faecalis* genomic DNA and various heterologous genomic DNAs into the *E. faecalis* enlarged protoplasts and investigated the effects of the injected DNAs on protoplast enlargement.

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**Table 1**  
Bacterial genomes used in microinjection into *Enterococcus faecalis* protoplasts.

Bacterial species	Gram staining	Genome size (Mbp)	GC content (%)
<i>Lactococcus lactis</i>	positive	2.6	35
<i>Enterococcus faecalis</i>	positive	2.8	37.5
<i>Lactobacillus curvatus</i>	positive	1.8	42
<i>Bacillus subtilis</i>	positive	4.2	43.5
<i>Escherichia coli</i>	negative	4.6	50.8
<i>Lelliottia amnigena</i>	negative	4.4	52.9
<i>Erythrobacter litoralis</i>	negative	3.3	65.2
<i>Deinococcus grandis</i>	negative	4.0	69.9

## 2. Materials and methods

### 2.1. Preparation and culture of protoplasts

*E. faecalis* NBRC 100480 was cultivated and protoplasts were prepared as previously described (Kami et al., 2019). The protoplasts were centrifuged at 7000 r.p.m. for 5 min and resuspended in Difco Marine Broth 2216 (DMB; 5 g/L peptone, 1 g/L yeast extract, 0.1 g/L ferric citrate, 19.45 g/L NaCl, 5.9 g/L MgCl<sub>2</sub>, 3.24 g/L MgSO<sub>4</sub>, 1.8 g/L CaCl<sub>2</sub>, 0.55 g/L KCl, 0.16 g/L NaHCO<sub>3</sub>, 0.08 g/L KBr, 34 mg/L SrCl<sub>2</sub>, 22 mg/L H<sub>3</sub>BO<sub>3</sub>, 8 mg/L Na<sub>2</sub>HPO<sub>4</sub>, 4 mg/L Na<sub>2</sub>SiO<sub>3</sub>, 2.4 mg/L NaF, and 1.6 mg/L NH<sub>4</sub>NO<sub>3</sub> [BD, Franklin Lakes, NJ]) containing 300 µg/mL penicillin G. 2 mL of DMB containing 300 µg/mL of penicillin G (Wako, Osaka) was added to the resulting suspension (10 µL) and incubated at 24 °C.

### 2.2. Microinjection workstation

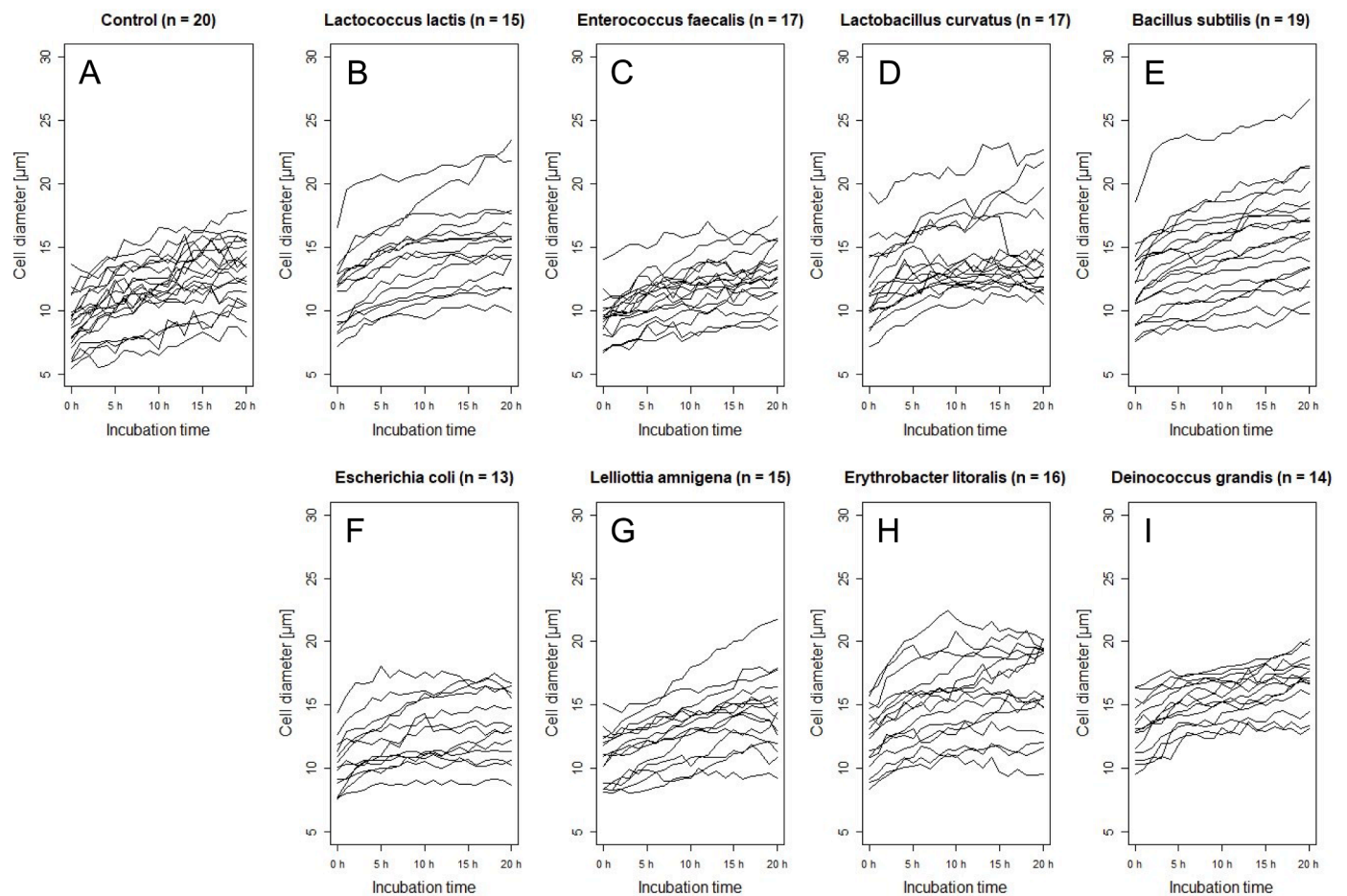
The microinjection workstation consisted of an Olympus IX73 (Olympus, Japan) differential interference microscope with a UPlanFL N objective 4×, a UPlanFL N objective 10×, a LUCPlan FL N objective 40×, and a UPlanFL N objective 60×. In addition, the microscope was equipped with a TransferMan 4r micromanipulator set (Eppendorf, Germany) with a FemtoJet 4i microinjector (Eppendorf, Germany).

### 2.3. Preparation of DNA injection solution

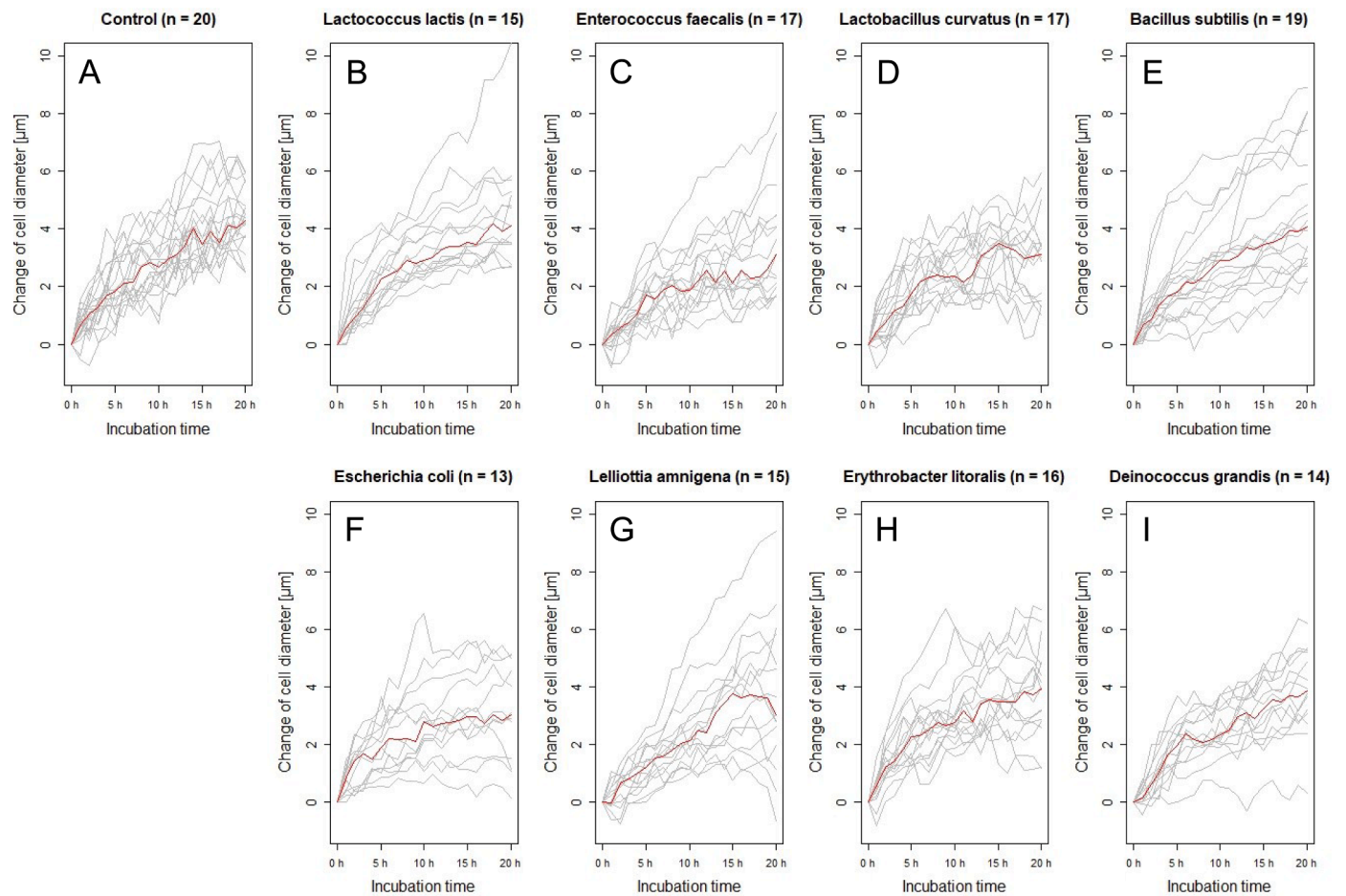
Heterologous genomic DNAs were selected in consideration of the evolutionary relationship and the DNA composition (GC content). Genomic DNAs of *D. grandis* ATCC 43672, *E. faecalis* NBRC 100480, *E. litoralis* NBRC 102620, *E. coli* MG 1655, *L. amnigena* NBRC 105700, *L. curvatus* NBRC 15884 and *L. lactis* NBRC 100933 were prepared using a DNeasy Blood and Tissue Kit (Qiagen, Germany) and were eluted with 25 mM Tris-HCl (pH 7.4) containing 300 mM sucrose. In addition, the genomic DNA of *B. subtilis* 168 was prepared using a modified method of Saito and Miura (1963) and was eluted with 25 mM Tris-HCl (pH 7.4) containing 300 mM sucrose. Finally, all bacterial genomic DNA was dissolved at a concentration of 100 ng/µL in 25 mM Tris-HCl (pH 7.4) containing 300 mM sucrose.

### 2.4. Loading the DNA injection solution into a microinjection needle

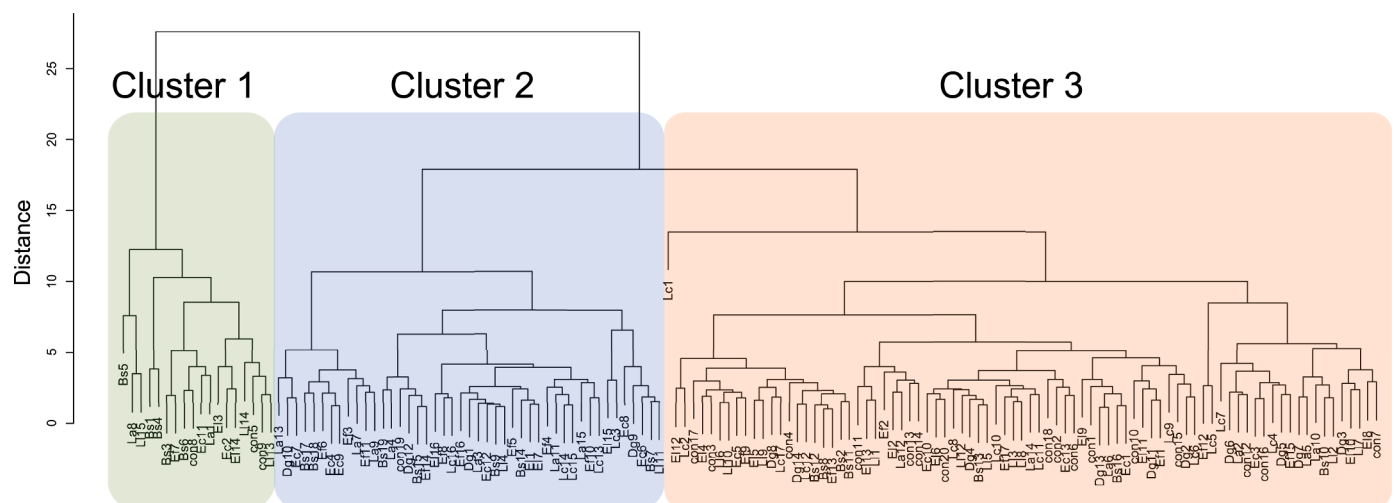
The DNA injection solution was mixed with ATP (Thermo Fisher Scientific Baltics UAB, Lithuania) at a final concentration of 5 mM



**Fig. 1.** Change of cell diameter of *E. faecalis* protoplasts. Control, no DNA injection. Bacterial species name means that its genomic DNA is injected at 0 h. The time-lapse observation was performed every hour for 20 h, using a BZ-X710 microscope (Keyence, Japan).



**Fig. 2.** Patterns of increase and decrease in cell diameter of *E. faecalis* protoplasts. Control, no DNA injection. Bacterial species name means that its genomic DNA is injected at 0 h. The time-lapse observation was performed every hour for 20 h, using a BZ-X710 microscope (Keyence, Japan). Red indicates a change of median at each incubation time.



**Fig. 3.** Phylogenetic relationships among 146 increasing and decreasing patterns in the cell diameter of *E. faecalis* protoplasts. The cluster analysis was performed using the statistical software R. The programs “dist” and “hclust” were used.

(Moriizumi et al., 2018) and centrifuged at  $11,000 \times g$  for 15 min at 4 °C. Next, a 2 µL aliquot was loaded into the tip of a Femtotip II microinjection needle (pore size: 0.5 µm, Eppendorf, Germany) using a micro-loader (Eppendorf, Germany). Next, the needle was tightly mounted in the capillary holder of the FemtoJet 4i microinjector, and then fixed

onto the micromanipulator.

### 2.5. Microinjection of *E. faecalis* enlarged cells

The *E. faecalis* enlarged protoplast culture (400 µL) at 48 h of

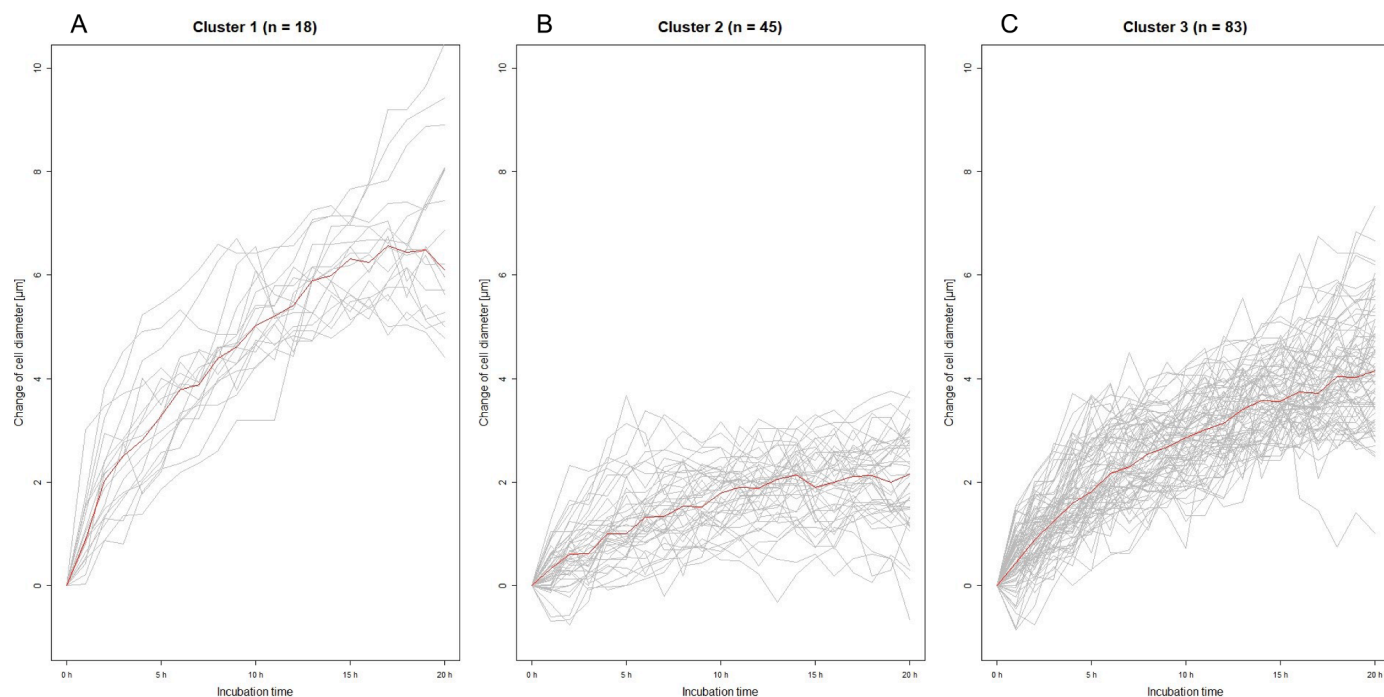


Fig. 4. Increasing and decreasing patterns in cell diameter of each cluster. Red indicates a change of median at each incubation time.

Table 2

P values in chi-square and Fisher’s exact tests.

Control vs.	Chi-square test	Fisher’s exact test
<i>Lactococcus lactis</i>	0.599	0.616
<i>Enterococcus faecalis</i>	0.00463	0.00223
<i>Lactobacillus curvatus</i>	0.0489	0.0476
<i>Bacillus subtilis</i>	0.0143	0.0146
<i>Escherichia coli</i>	0.0148	0.0120
<i>Lelliottia amnigena</i>	0.0128	0.0123
<i>Erythrobacter litoralis</i>	0.226	0.309
<i>Deinococcus grandis</i>	0.0710	0.0883

incubation in DMB containing penicillin G was mounted on a cover glass (40 × 50, Matsunami, Japan) and placed on the microscope stage. First, the DNA solution was released into enlarged cells using the FemtoJet 4i microinjector. Next, Femtotip II was inserted into enlarged cells for approximately 10–60 s while the DNA solution was released at 50–100 hPa. An intracellular change confirmed successful injection. Following

the microinjection, the cover glass was put in a petri dish, and a time-lapse observation was performed under a BZ-X710 microscope (Keyence, Japan).

2.6. Cell size measurement

Bright-field microscopy images of the protoplasts were obtained using the BZ-X710 microscope (Keyence, Japan). The cell sizes were measured using the cellSens Standard 1.11 imaging software (Olympus, Tokyo).

2.7. Computer analyses

Each profile of cell diameters from 0 to 20 h was used for cluster analysis. The programs “dist” and “hclust” were used in the statistical software R (<http://www.R-project.org/>). Two statistical tests, namely, the chi-square test and Fisher’s exact test, were also performed using R.

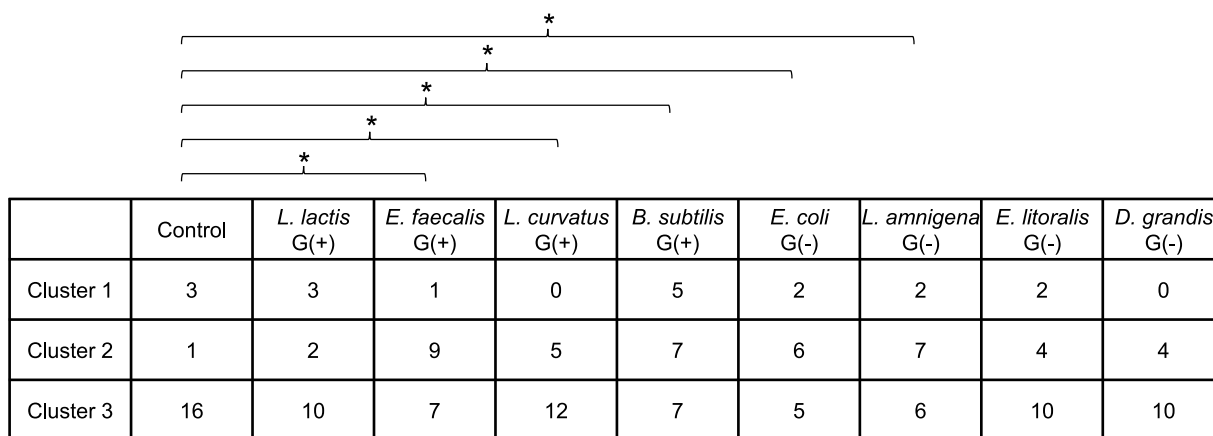


Fig. 5. The number of protoplasts at each cluster of different bacterial genomic DNA injections. Chi-square and Fisher’s exact tests were performed using the statistical software R. \*, p < 0.05; G(+), Gram-positive; G(-), Gram-negative.

### 3. Results and discussion

#### 3.1. *E. faecalis* protoplast enlargement patterns were clustered into three groups

The bacterial genomic DNAs used in this study are shown in Table 1. The *E. faecalis* protoplasts that the *E. faecalis* genomic DNA or heterologous genomic DNAs were microinjected into, continued to enlarge (Fig. 1). The 126 protoplasts of *E. faecalis* into which eight phylogenetically distant bacterial genomic DNAs were injected had been observed by time-lapse microscopy (every hour for 20 h). These enlargement patterns were compared with 20 *E. faecalis* protoplasts (control, Fig. 1A) that were not treated by microinjection. Thus, most *E. faecalis* protoplasts possessing injected genomic DNAs can survive.

We compared patterns of increase and decrease in cell diameter of those protoplasts (Fig. 2) and performed cluster analysis. As a result, a total of 146 enlargement patterns were clustered into three groups (Fig. 3). Cluster 1 consists of 18 protoplasts that have the highest enlargement level in the three clusters (Fig. 4A). Cluster 2 consists of 45 protoplasts that have the lowest enlargement level among the three clusters (Fig. 4B). Finally, cluster 3 consists of 83 protoplasts, in which 16 of 20 control protoplasts existed (Fig. 4C).

#### 3.2. *E. faecalis* genomic DNA injection weakened the protoplast enlargement

The chi-square and Fisher's exact tests indicated that the protoplast enlargement patterns of the cells into which *E. faecalis* genomic DNA (Figs. 1C and 2C) was injected differed significantly ( $p < 0.05$ ) from those of the control *E. faecalis* protoplasts (Table 2, Fig. 5). Thus, clusters 1 and 3 decreased, and cluster 2 increased (compared between "Control" and "*E. faecalis*" in Fig. 5), indicating that the protoplast enlargement was weakened by injecting its own genomic DNA into *E. faecalis* protoplasts.

Our previous studies showed that *E. faecalis* protoplast enlargement requires DNA replication (Kami et al., 2019; Tsuchikado et al., 2020). DNA attachment to the plasma membrane (Earhart et al., 1968; Leibowitz and Schaechter, 1975; Marvin, 1968; Sueoka and Quinn, 1968) might be needed for enlargement. If so, the genomic DNA injection leads to enlargement promotion. In addition, the RNA expression from the *E. faecalis* genome may require enlargement. Therefore, the *E. faecalis* genome injection should promote enlargement. However, *E. faecalis* genomic DNA injection into *E. faecalis* protoplasts weaken the enlargement. Thus, these are considered unlikely possibilities.

In our opinion, the DNA replication system is associated with plasma membrane biosynthesis. Therefore, the injected *E. faecalis* genomic DNA may weaken DNA replication, and then plasma membrane biosynthesis may be weakened in conjunction with it. Furthermore, the injected *E. faecalis* genomic DNA may include fragmented DNA. This fragmented DNA may interfere with the replication of the *E. faecalis* intact chromosome. More work is needed to elucidate the interference.

#### 3.3. Microinjection of four heterologous genomic DNAs weakened the enlargement of *E. faecalis* protoplasts

Microinjection of the Gram-positive bacterial genomes of *Bacillus subtilis* and *Lactobacillus curvatus* (Figs. 1D, 1E, 2D, and 2E) and the Gram-negative bacterial genomes of *Escherichia coli* and *Lelliottia amnigena* (Figs. 1F, 1G, 2F, and 2G) into *E. faecalis* protoplasts led to a weakened enlargement as well as that of the *E. faecalis* genome ( $p < 0.05$ ; Table 2, Fig. 5). Conversely, microinjection of the Gram-positive *Lactococcus lactis* genome (Figs. 1B and 2B) and the Gram-negative *Deinococcus grandis* and *Erythrobacter litoralis* (Figs. 1H, 1I, 2H, and 2I) did not lead to enlargement patterns different from that of the control ( $p > 0.05$ ; Table 2, Fig. 5). Therefore, considering that *E. faecalis* is Gram-positive, the effects of heterologous genomic DNA injection on

protoplast enlargement are independent of bacterial evolutionary relationships.

The GC content of the *E. faecalis* genome is 37.5%. Only the *L. lactis* genome has the lowest GC content (35%) among the seven heterologous genomes. The effect of microinjection of the *L. lactis* genome on enlargement did not differ from that of the control ( $p > 0.05$ ; Table 2, Fig. 5). The *B. subtilis*, *E. coli*, and *L. amnigena* genomes have a higher GC content than the *E. faecalis* genome. The effects of microinjection of these heterologous genomes on enlargement differed from those of the control ( $p < 0.05$ ; Table 2, Fig. 5). These results suggest that genomic DNAs with GC content higher than that of the *E. faecalis* genome may function in the *E. faecalis* protoplasts. However, microinjection of the *D. grandis* and *E. litoralis* genomes with GC content much higher than that of the *E. faecalis* genome did not have different effects from that of the control ( $p > 0.05$ ; Table 2, Fig. 5). Therefore, it is uncertain whether the GC content of microinjected genomic DNA affects protoplast enlargement or not.

When the injected DNAs are aggregated in the protoplasts, the injected DNA may not influence protoplast enlargement. When the injected DNAs are not aggregated, the DNA may function in the protoplasts. However, if the injected DNA hybridizes to the host *E. faecalis* chromosome, the chromosome replication may be weakened. This replication weakness may influence plasma membrane biosynthesis. Moreover, even if the injected genomic DNAs do not hybridize to the host chromosome, the heterologous genomic DNAs might reduce the replication efficiency of the host *E. faecalis* chromosome.

### 4. Conclusion

We generated more than 100 *E. faecalis* protoplasts into heterologous genomic DNAs. Protoplast enlargement did not stop and continued after the microinjection. The effect of the microinjected genome was observed, which was species-dependent but did not reflect bacterial phylogeny. The effect is that microinjection of genomic DNA weakened enlargement. We clarified the difference in the effect of the injected genomic DNA on enlargement, but it is uncertain how the injected DNA exists in the host cell. More work is needed to elucidate whether the DNA sequence plays an important role in the weakness of protoplast enlargement.

#### Credit Author Statement

Sawako Takahashi: Validation, Formal analysis, Data curation, Writing-original draft

Hiroshi Nishida: Conceptualization, Formal analysis, Data curation, Writing-original draft, Writing-review and editing, Supervision, Project administration.

#### Declaration of Competing Interest

The authors declare that they have no competing interests.

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