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RESEARCH ARTICLE

Cell-to-cell non-conjugative plasmid transfer between *Bacillus subtilis* and lactic acid bacteria

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Abstract

Bacillus subtilis is a soil-dwelling bacterium that can interact with a plethora of other microorganisms in its natural habitat. Due to the versatile interactions and its ability to form nanotubes, i.e., recently described membrane structures that trade cytoplasmic content between neighbouring cells, we investigated the potential of HGT from *B. subtilis* to industrially-relevant members of lactic acid bacteria (LAB). To explore the interspecies HGT events, we developed a co-culturing protocol and provided proof of transfer of a small high copy non-conjugative plasmid from *B. subtilis* to LABs. Interestingly, the plasmid transfer did not involve conjugation nor activation of the competent state by *B. subtilis*. Moreover, our study shows for the first time non-conjugative cell-to-cell intraspecies plasmid transfer for non-competent *Lactococcus lactis* sp. *cremoris* strains. Our study indicates that cell-to-cell transformation is a ubiquitous form of HGT and can be potentially utilized as an alternative tool for natural (non-GMO) strain improvement.

INTRODUCTION

Horizontal gene transfer (HGT) plays a crucial role in bacterial evolution and ecology. In adaptation to changing environments, bacteria acquire foreign DNA that may offer beneficial properties under various selection pressures (Cohan & Koeppel, 2008; Wiedenbeck & Cohan, 2011). Prevalent evidence of the ecological importance of HGT is the acquisition and spread of antibiotic resistance and virulence determinants in closely related species, which can have significant consequences for the emergence of antibiotic-resistant pathogens (Deng et al., 2019; Schmidt & Hensel, 2004; Siddaramappa et al., 2011).

The generally recognized and well-known mechanisms of HGT in bacteria are conjugation, transduction, and natural transformation. In the transformation process, bacteria enter a transient state of competence and activate the assembly of the DNA-uptake machinery to acquire naked DNA directly from the environment (Chen et al., 2005; Chen & Dubnau, 2004; Lorenz & Wackernagel, 1994). This process is genetically encoded in the recipient's cell, and it is regulated by many physiological and environmental factors such as growth phase, nutrient availability, starvation, cell density, and even antibiotic stress (Claverys et al., 2006; Dubnau, 1991a; Hamoen et al., 2003; Håvarstein et al., 1996; Slager et al., 2014). While the DNA-uptake machinery is generally conserved across species, the induction conditions and type of inducers vary between species. For instance, *Bacillus subtilis* induces competence at the onset of the stationary phase or in response to high cell density (Anagnostopoulos & Spizizen, 1961; Dubnau, 1991b). In contrast, in *Streptococcus pneumoniae*, competence activation was observed at a specific time during the exponential phase and was inhibited at the stationary phase (Håvarstein et al., 1995). Notably, many bacteria display a complete set of competence-induced genes; however, the transformation-promoting conditions are still not always elucidated (David et al., 2017; Mulder et al., 2017; Wydau et al., 2006).

Unlike natural transformation, conjugation and transduction require the presence of a donor cell, or a donor

phage, respectively, and engage specific conduits for DNA exchange. In the case of conjugation, after the mating pair formation between a compatible donor and recipient, the genetic material is transferred through a mating channel or conjugative pilus, typically encoded on the conjugative elements (Auchtung et al., 2016; Cabezón et al., 2015). During transduction, genetic information is carried through phage virions that acquired random fragments of the host's DNA during capsid assembly and were able to infect both donor and recipient (Chiang et al., 2019; Clokie et al., 2011; Marcelli et al., 2020). While conjugation and transduction certainly contribute to HGT in bacteria, their transfer capacity is limited by many factors, including surface exclusion, type and size of genetic material, host-range specificity, restriction-modification systems present in the recipient, and sexual isolation (Dahmane et al., 2017; Mahony et al., 2017; Majewski, 2001; Thomas & Nielsen, 2005).

Besides these classical HGT mechanisms, recent evidence support the idea of alternative means of DNA transfer in bacteria, including membrane vesicles (Domingues & Nielsen, 2017), cell-to-cell natural transformation (Blesa et al., 2015; Etchuuya et al., 2011; Matsumoto et al., 2016; Zhang et al., 2018), and nanotubes (Dubey & Ben-Yehuda, 2011). Interestingly, *B. subtilis* has been shown to transfer genetic material not only via conjugation (Grohmann, 2010a, 2010b; Lee et al., 2012; Rösch et al., 2014; Singh et al., 2013) or phage transduction (Deichelbohrer et al., 1985; Tzipilevich et al., 2017; Yasbin & Young, 1974) but also through cell-to-cell non-conjugative plasmid transfer (Dubey & Ben-Yehuda, 2011; Zhang et al., 2018), which makes it an excellent candidate to study intra- and interspecies HGT events. *B. subtilis* and members of lactic acid bacteria (LAB) are of great importance in food and feed production and the probiotic market. However, the current strain improvement methods for the production of new starter cultures are limited due to public resistance and reinforced by the European Union directives on the use of genetically modified organisms. Thus, in recent years, the interest in naturally occurring HGT processes for the mobilization of genetic traits has been renewed (Bron et al., 2019; Kuipers, 2015; Marcelli et al., 2020). Here, we explore the potential of HGT within co-cultures of *B. subtilis* and industrially-relevant LAB, including *Lactococcus lactis* and *Streptococcus thermophilus*. For the first time, we demonstrate the inter- and intraspecies transfer of a high copy, non-conjugative plasmid in non-competent and conjugation-negative *B. subtilis* and *L. lactis* strains with co-culturing techniques.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

B. subtilis strains were grown at 37°C in Lysogeny Broth (LB), either aerated by shaking at 220 rpm or on solidified LB with 1.5% (w/v) agar; *L. lactis* strains were cultured at 30°C in Difco M17 medium supplemented with glucose at the final concentration 0.5% (w/v) (GM17), as standing cultures or on solidified GM17 with 1.5% (w/v) agar. *S. thermophilus* strains were grown anaerobically as standing cultures at 37°C in Difco M17 medium supplemented with glucose (0.5% w/v) and lactose (1% w/v) (GLM17) or on solidified GLM17 with 1.5% (w/v) agar. When needed, growth media were supplemented with antibiotics at the following final concentrations: chloramphenicol (cm) 5 μg/ml, tetracycline (tet) 5 μg/ml, spectinomycin (spec) 100 μg/ml, and erythromycin (ery) 5 μg/ml.

All strains used in this study are listed in Table 1. All plasmids and oligonucleotides used are listed in Tables 2 and 3, respectively.

Construction of *Lactococcus lactis* strains lacking the sex factor

For the knock-out of *cluA* in *L. lactis* MG1363, the pGh9 thermosensitive plasmid system, a gift from Dr. Saulius Kulakauskas, was used. Transformation of electrocompetent *L. lactis* MG1363 with the pGh9 plasmid containing the *cluA* sequence was performed as previously described (Maguin et al., 1996). In *L. lactis*, pGh9 replicates at 28°C, but it is lost when cells are cultured at temperatures above 37°C. The pGh9-*cluA* plasmid derivative was used to integrate the plasmid in the native *cluA* locus at the permissive temperature of 28°C. Further excision of the plasmid at 37°C resulted in the *L. lactis* MG1363 Δ*cluA* strain.

Mating procedure—cell-to-cell plasmid transfer

Monocultures of donor and recipient were inoculated from an overnight culture, diluted to a final OD_{600} of 0.05, supplemented with an appropriate antibiotic, and incubated at the optimal temperature separately: 30°C for *L. lactis* and 37°C for *B. subtilis*. Subsequently, cultures were grown until they reached mid-log growth phase (OD_{600} ~0.5). After the incubation period, cells were harvested and washed in 1XSMM (Harwood & Cutting, 1990) media to a final density of 10^7 cells per 1 μl of culture. Donor and recipient cells were mixed in the cell ratio 1:1 (donor: recipient), and 5 μl of mixed culture was spotted on nonselective GM17 medium, air-dried, and incubated O/N at the optimal temperature: 37°C for *B. subtilis* matings and 30°C for *L. lactis* matings only. For *S. thermophilus*, matings were performed on GM17 media supplemented with 1% lactose (GLM17), and the co-culture was incubated at 37°C. Cells from the O/N mating plates were harvested using 1 ml of 1XSMM medium and plated on selective rich medium plates with appropriate antibiotics and

TABLE 1 Strains used in this study.

Strain	Species	Genotype	Ab^r	Reference
PY79	*B. subtilis*	Prototroph SPβ, ICE*Bs1*$^-$	–	BGSC
PY79 Δ*comK*	*B. subtilis*	PY79; *comK*::spec	spec	This work
PY79 pNZ8048	*B. subtilis*	PY79; pNZ8048	cm	This work
168	*B. subtilis*	trpC2	–	BGSC
168 pNZ8048	*B. subtilis*	168 trpC2, pNZ8048	–	BGSC
168 Δ*comK*	*B. subtilis*	168 trpC2, *comK*::spec	spec	Laboratory stock
168 Δ*amyE*	*B. subtilis*	168 trpC2, *amyE*::tet	tet	Laboratory stock
MG1363	*L. lactis*	Plasmid-free derivative of NCDO712 (Prt-, Lac-)	–	Gasson (1983)
MG1363 GFP$^+$	*L. lactis*	MG1363; *pseudo10*::P*usp45-sfGFP*(Bs)	ery	Overkamp et al. (2013)
MG1363 Δ*cluA*	*L. lactis*	MG1363; Δ*cluA*	–	This work
MG1363 GFP$^+$ Δ*cluA*	*L. lactis*	MG1363_sfgfp(Bs); Δ*cluA*	ery	This work
MG1363 pNZ8048	*L. lactis*	MG1363; pNZ8048	cm	This work
MG1363 Δ*cluA* pNZ8048	*L. lactis*	MG1363; Δ*cluA*, pNZ8048	cm	This work
MG1363 pNZ521	*L. lactis*	MG1363; pNZ521	cm	This work
MG1363 Δ*cluA* pNZ521	*L. lactis*	MG1363; Δ*cluA*, pNZ521	cm	This work
CNRZ302 pNZ8048	*S.thermophilus*	pNZ8048	cm	This work
ST11 pNZ8048	*S.thermophilus*	pNZ8048	cm	This work

TABLE 2 Plasmids used in this study.

Plasmid	Genotype	Ab^r	Reference
pNZ8048	NICE inducible vector	cm	de Ruyter et al. (1996)
pseudo10::Pusp45-sfGFP(Bs)	Integrative vector pSEUDO10 with Pusp45-sfGFP(Bs) sequence	ery	Overkamp et al. (2013)
pGh9::*cluA*	pGh9 thermosensitive plasmid (a replication thermosensitive derivative of pWV01; Otto et al., 1982) with *L. lactis cluA* construct for deletion, cloned by Gibbson assembly to pGh9 through SmaI, unpublished	ery	Strain collection of Dr. Kulakauskas (unpublished)
pNZ521	10.7 kb pNZ122 derivative carrying complete *prtP* and *prtM* gene of pSK111	cm	Marugg et al. (1995)

TABLE 3 List of primers used in this study.

Name	Sequence 5′→3′	Amplified fragment	Product size (bp)
cmR_FW	GCAGACAAGTAAGCCTCCTA	1	767
cmR_RV	GGGGCAGGTTAGTGACATTAGA	1	
repA_FW	TGCGGCGTTAGCTATAGAAG	2	1225
repA_RV	CTGCTTTCTTCATTAGAATCAATC	2	
Pnis_FW	CCAAGATCTAGTCTTATAACTATAC	3	803
Pnis_RV	CGGCTTTCATAATCTAACAGAC	3	
repC_FW	TATGAAAGCCGATGACTGAATG	4	539
repC_RV	AACCGCAGATTTTGAAAAAC	4	

subsequently incubated at the indicated temperature for O/N. Plates with transformants were replicated on rich selective medium, GM17 for *B. subtilis* and *L. lactis* and GLM17 for *S. thermophilus*, with appropriate antibiotics to exclude the nonheritable transfer of chloramphenicol acetyltransferase in case of chloramphenicol resistance. After the overnight incubation, transformants were propagated in selective liquid medium and

plasmids were isolated (NucleoSpin Plasmid, Mini kit for plasmid DNA, Macherey-Nagel, GmbH & Co. KG, Germany) for gel electrophoresis (horizontal gel electrophoretic system from Bio-Rad Laboratories, Inc., The Netherlands). Additionally, to confirm the presence of pNZ8048 in *L. lactis* transformants, a PCR reaction (T100 Thermal Cycler, Bio-Rad Laboratories, Inc., The Netherlands) with pNZ8048 annealing primers (Table 3) was performed.

DnaseI treatment

DnaseI treatment was performed similarly to previously published work on nanotubes (Dubey & Ben-Yehuda, 2011). The donor and recipient strains were separately grown to the mid-exponential phase, subsequently pelleted and washed with DNaseI buffer (50 mM Tris, pH 7.2, 10 mM $MgCl_2$, 5 mM $CaCl_2$), and incubated separately in the presence of 100 μg/ml of DNaseI for 15 min at 37°C. Donor and recipient were mixed in a 1:1 ratio, and the mixtures were supplemented with DNaseI (100 μg/ml) and spotted on LB agar. DNaseI buffer (without DNaseI) was added as a control. Mixed co-cultures were grown for 18 h at 30°C or 37°C and replica plated on respective antibiotic plates.

Microscopy techniques

TIRF microscopy—membrane visualization with FM5-95

Cultures were prepared as above and spotted on 1% (w/v) agarose in GM17 supplemented with FM5-95 membrane dye at a final concentration of 1 μg/ml and incubated at 37°C for 1 h prior to the experiment. For the visualization of nanotubes at high resolution, the TIRF 60× objective was used (Olympus 60X/1.49, APON 60XOTIRF, UIS2, 1-U2B720). To observe membrane structures with FM5-95 dye, a filter set Quad-mCherry was used: excitation with laser at 568 nm and emission at 594 nm.

SEM

In mixed cultures, *B. subtilis* and *L. lactis* were grown separately in rich media to mid-exponential growth phase and mixed in 1:1 ratio to visualize nanotubes in mixed cultures. The mixture was spotted on GM17 agar, and EM copper grids were placed on top of it and incubated for 6 h at 37°C. After the incubation period, cells were fixed with glutaraldehyde (2%) for overnight at 4°C. The next day, cells were washed in 0.1 M sodium cacodylate buffer and incubated with 1% osmium tetroxide (prepared in 0.1 M sodium cacodylate) for 1 h at room temperature. Post-fixed sample was washed 3 times with water and subsequently dehydrated with 30%, 50%, and 70% ethanol for 15 min and finally with 100% ethanol (3 × 30 min). Last, samples were incubated in 100% ethanol:tetramethylsilane (TMS) (1:1). After 10 min, incubation samples were treated with pure TMS for 15 min and subsequently air-dried prior imaging.

RESULTS

B. subtilis, *L. lactis* and *S. thermophilus* are able to form membranous structures that resemble bacterial nanotubes

Visualization of membranous structures that might facilitate HGT in studied mixed co-cultures

In the recent decade, the presence of membranous structures bridging neighbouring bacterial cells was extensively studied (Bhattacharya et al., 2019; Dubey et al., 2016; Dubey & Ben-Yehuda, 2011; Pal et al., 2019; Pande et al., 2015; Stempler et al., 2017). With the use of microscopic techniques, these membrane connections have been identified and characterized in Gram-positive (*B. subtilis*, *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. It has been proposed that structures like nanotubes can conduit the HGT events in an intra- and interspecies manner (Dubey & Ben-Yehuda, 2011). Prompt by these recent findings, we wondered whether these membranous connections are present in *L. lactis*, *S. thermophilus*, and more importantly, we explored interspecies interactions of these microorganisms with *B. subtilis*—a known nanotube producer, in mixed co-culutres.

Accordingly, we optimized co-culturing conditions for chosen microorganisms and performed microscopic analysis in tested environment. We observed that *B. subtilis* and *L. lactis* can co-exist when spotted on rich medium GM17 and co-incubated at 37°C. For *B. subtilis* and *S. thermophilus*, the optimal conditions were GM17 with 1% of lactose and incubation at 37°C. Given the environment, we examined co-cultures after 6 h incubation with scanning electron microscopy (SEM) and visualized inter- and intraspecies membrane connections, nanotubes, known to facilitate molecular transfer events, using total internal reflection fluorescence microscopy (TIRF) (Figure 1 and Supplementary Materials).

Non-conjugative plasmids can be transferred between *B. subtilis* and members of LAB

The microscopy analysis of mono and co-cultures of each studied microorganism indicates that cells grown

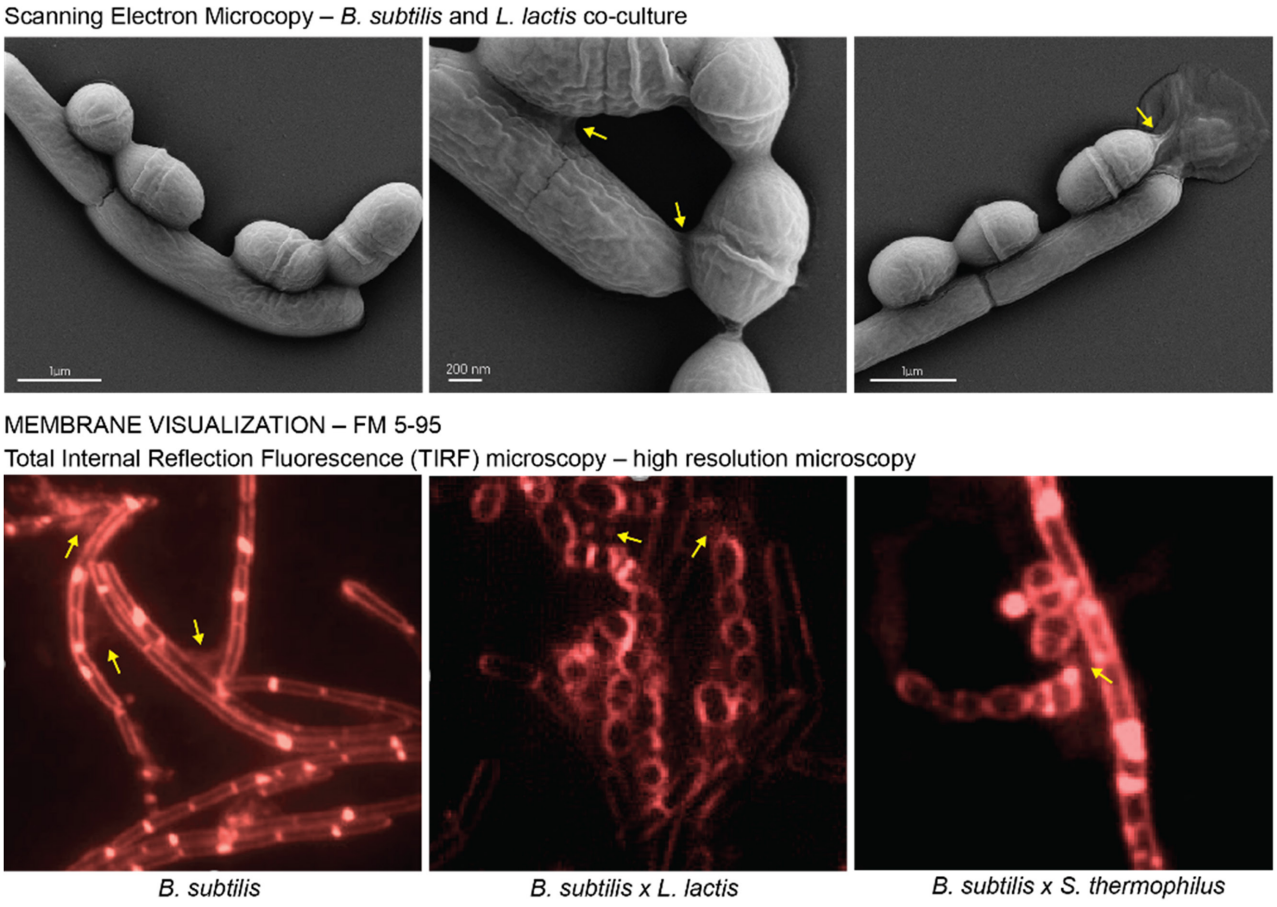

FIGURE 1 SEM of *B. subtilis* PY79 and *L. lactis* MG1363 after 6 h of co-culture. Top row: Scanning electron microscopy of *B. subtilis* PY79 and *L. lactis* MG1363 co-cultured for 6 h on solid GM17 media show that *B. subtilis* can co-exist with *L. lactis* in close proximity. The close contact places are marked with yellow arrows. Bottom row: micrographs of FM5-95 membrane staining of studied co-cultures reveal the presence of membranous connections (yellow arrows) similar to previously reported bacterial nanotubes.

on solid rich media produce membranous structures that resemble bacterial nanotubes (Figure 1). Since the occurrence of membrane connections might facilitate HGT and so-called cell-to-cell transformation, we examined interspecies plasmid transfer between *B. subtilis* and LAB members and designed a collection of donor strains harbouring high-copy, nonconjugative shuttle vector pNZ8048 (3.4 kb, cm^R) replicating in both, donor and recipient cells (de Ruyter et al., 1996). To facilitate further selection of newly engineered strains, recipient strains contained a chromosomally integrated antibiotic cassette (Experimental Procedures; Table 1).

Plasmid transfer between *B. subtilis* strains

First, we investigated cell-to-cell transformation between two *B. subtilis* strains. For the *B. subtilis* matings, we started with a well-characterized *B. subtilis* 168 laboratory strain. Since *B. subtilis* is able to internalize free DNA from its environment upon induction of the competence state, we decided to use a recipient strain with a knocked-out *comK* gene, encoding the competence master regulator ComK (Hahn et al., 1996; van Sinderen et al., 1994), to ensure that during the mating procedure the transferred plasmid was not taken up by the recipient cell from adjacent lysed donor cells. Additionally, the *comK*⁻ strain carried a spectinomycin antibiotic cassette for later selection on LB agar plates. For the donor strain, we used *B. subtilis* 168 harbouring the pNZ8048 (cm^R) non-conjugative shuttle plasmid. To examine the plasmid transfer, we spotted a mix of donor and recipient cells on a rich non-selective agar plate and incubated overnight without antibiotic selection pressure. As a control, we spotted donor and recipient cells separately on the same type of non-selective agar plates. To additionally exclude DNA uptake via activation of the natural competence and confirm the cell-to-cell DNA transfer, we separately spotted recipient cells on the mating plates and added 1 µg of exogenous pNZ8048 DNA. After the incubation time, the formed mixed macrocolonies were collected, and the recipient strain's antibiotic resistance was tested. The analysis of selectable plates showed that all colonies resistant to both antibiotics, $spec^R$ and cm^R, were

derived only from the mixed population of donor and recipient (Figure 2A). We did not observe any resistant colonies of monocultures of donor or recipient on selectable media, indicating that recipient cells should be in the same environment as the donor cells in order to form colonies on spectinomycin and chloramphenicol agar plates. Moreover, the recipient strain could not take up externally provided plasmid DNA from the media, which excluded the possibility of natural plasmid transformation. After replica plating, the colonies obtained were additionally examined for plasmid presence (Figure 2B). The transfer efficiency was estimated as a fraction of recipients that received the genetic material from donor cells and was estimated on 10^{-2} transformants per CFU recipient (Figure 2C).

To further investigate the relevance of functional natural competence in the cell-to-cell transformation, we compared plasmid transfer efficiencies when used 168 Δ*comK* (spec^R) strain 168 Δ*amyE* (tet^R), *comK*+ strain as recipient strains. It is worth mentioning that lack of a functional *amyE* gene, encoding an alpha-amylase used by *B. subtilis* to degrade starch, should not affect the transfer efficiency or diminish the efficiency of natural transformation on a solid medium. Following the mating procedure, the same donor strain of *B. subtilis* 168 pNZ8048 was co-cultured with either 168 Δ*comK* (spec^R) or 168 Δ*amyE* (tet^R) on solid media and the plasmid transfer was examined accordingly (Figure 2A). On average, the efficiency of plasmid transfer to 168 Δ*amyE* (tet^R) was not significantly different when compared with the 168 Δ*comK* (cm^R) (Figure 2C), demonstrating that under the mating conditions, the putative activation of natural competence in *B. subtilis* cells does not facilitate plasmid acquisition.

Genome analysis of the *B. subtilis* 168 strain has revealed the presence of the temperate phage SPß (Warner et al., 1977) and the integrative and conjugative element ICE*Bs1* (Auchtung et al., 2005, 2007). Therefore, to ensure that the plasmid transfer is cell-to-cell contact-dependent and not a result of conjugative events or induced prophage transduction, we tested the *B. subtilis* PY79 strain, cured from ICE*Bs1* and SPβ. Subsequently, we designed a competence negative strain of PY79 by inserting spectinomycin antibiotic cassette in the *comK* gene, resulting in *B. subtilis* PY79 Δ*comK* (spec^R) recipient strain. For the donor strain, we used *B. subtilis* PY79 pNZ8048 (cm^R) strain. Estimated transfer efficiencies revealed that PY79 performed similarly to the 168 strain, indicating that pNZ8048 was transferred in a non-conjugative manner, and it did not involve phage transduction (Figure 2C). Additionally, we found that plasmid transfer was resistant to DNaseI, showing that pNZ8048 was likely transferred via protective conduit rather then taken up directly from the environment. Transfer efficiency for *B. subtilis* matings was estimated to yield 10^{-2} transformants per CFU recipient (Figure 2C).

Interspecies plasmid transfer between *B. subtilis* and *L. lactis*

L. lactis is a Gram-positive bacterium widely used as a starter culture for milk fermentation, a host for heterologous protein production and a platform for delivery of therapeutics. Therefore, the ability to acquire new genetic traits by omitting the genetic engineering steps would greatly contribute to the design and production of novel strains with industrially relevant features. Because of several mutations in competence-related genes, *L. lactis* MG1363 is not able to enter the competence state (Mulder et al., 2017). Therefore, it was possible to study cell-to-cell transformation, excluding DNA take-up from the environment.

B. subtilis to *L. lactis* plasmid transfer

Given that *B. subtilis* can efficiently deliver non-conjugative plasmids to its neighbours, we designed our first mating experiment exploiting interspecies plasmid transfer between *B. subtilis* and *L. lactis*. As a donor strain, we used *B. subtilis* 168 pNZ8048 (cm^R) and PY79 pNZ8048 (cm^R), and as a recipient strain, we used *L. lactis* MG1363 transformed with pSEUDO10_*sfgfp*(Bs) (ery^R) (MG1363 GFP$^+$). Accordingly, donor and recipient strains were co-cultured on solid rich media supporting growth of both strains (GM17) at 37°C, and the antibiotic resistance of the recipient strain after mating was analysed. Since the reciepient strain harboured an erythromycin cassette integrated into its genome, selection of *L. lactis* transformants was performed on erythromycin (5 μg/ml) and chloramphenicol (5 μg/ml) GM17 plates. The cell-to-cell plasmid transfer yielded many *L. lactis* MG1363 colonies resistant to 5 μg/ml of erythromycin and 5 μg/ml of chloramphenicol after the first selection round (Figure 3A), and the plasmid transfer efficiency was estimated on 10^{-4} to 10^{-5} transformants per CFU recipient. However, we could not propagate the transformants obtained in the liquid media or solid selectable rich media (GM17, erythromycin 5 μg/ml, chloramphenicol 5 μg/ml). Interestingly, the replica plating of the transformants on rich media with reduced chloramphenicol concentrations to 1 μg/ml and 2.5 μg/ml resulted in *L. lactis* single colonies as well as a few colonies with *B. subtilis* morphology (Figure 3B). To additionally confirm the identity of the obtained colonies classified as *L. lactis* transformants, we performed a microscopy analysis and confirmed that these colonies are MG1363 GFP$^+$ cells (Figure 3C).

Next, we selected single colonies of MG1363 GFP$^+$ pNZ8048, inoculated in GM17 with 5 μg/ml of erythromycin and 2.5 μg/ml of chloramphenicol and

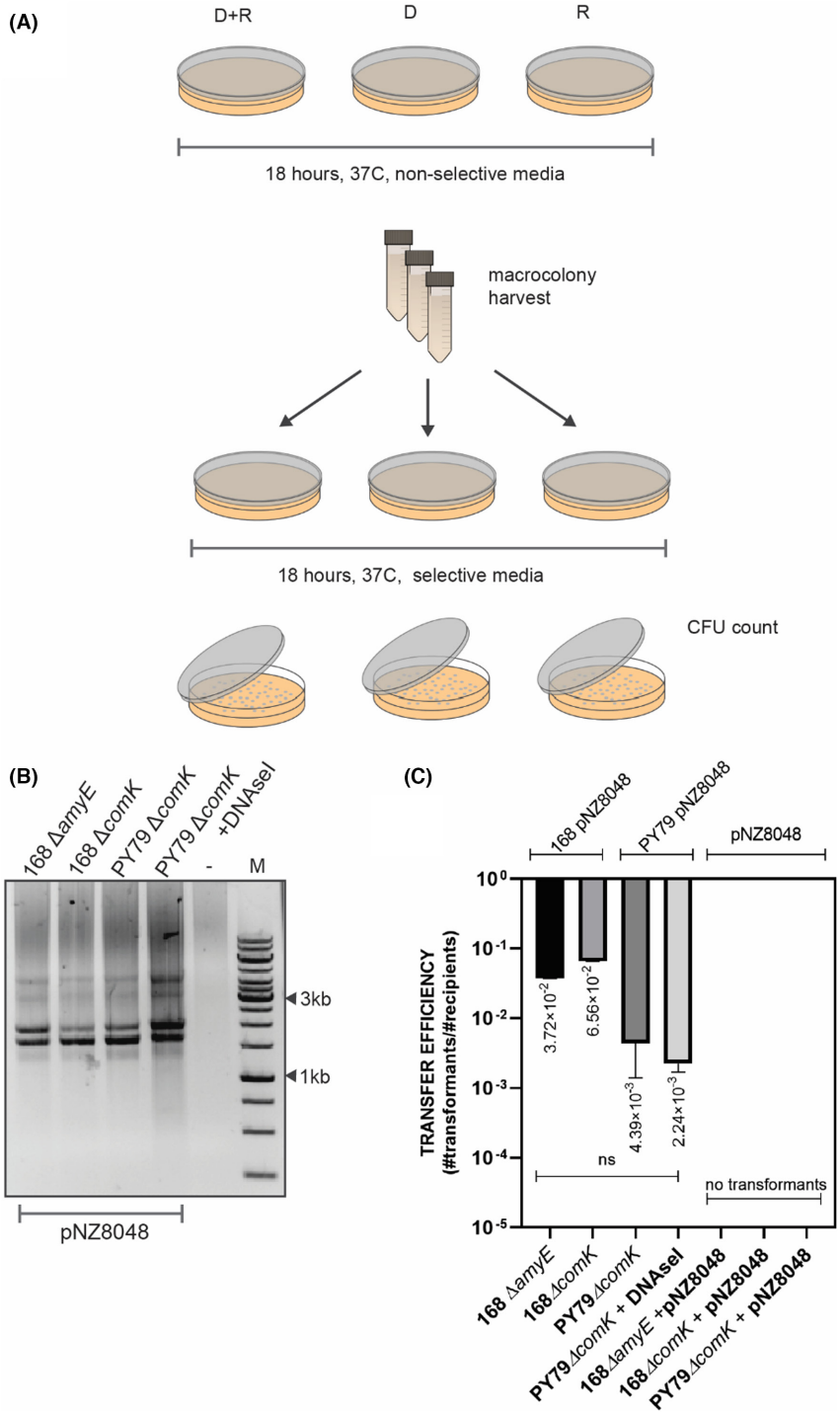

FIGURE 2 Cell-to-cell transformation of nonconjugative plasmid between *B. subtilis* strains. (A) A graphic representation of mating procedure. (B) Gel electrophoresis of isolated pNZ8048 from the recipient strain after the replica plating. (C) The average values of plasmid transfer efficiency for *B. subtilis* to *B. subtilis* matings. Donors of the genetic material are indicated on top of the graph. Mating without donor strain but with exogenously added pNZ8048 did not yield in colonies (indicated with 0). The statistical analysis was performed in Prism (Kruskal–Wallis test, ns, not significant).

verified the presence of pNZ8048 in the *L. lactis* culture by plasmid extraction and by direct colony PCR. The gel electrophoresis of isolated pNZ8048 from transformed cells did not show pNZ8048-specific bands (Figure 3D). Conversely, PCR analysis of the MG1363 GFP$^+$ pNZ8048 colony and genomic DNA isolated from the same colony revealed the presence of pNZ8048 fragments, including chloramphenicol cassette (Figure 3E), suggesting that colonies from replica plating likely harboured copies of pNZ8048, however in a reduced amount.

L. lactis to *B. subtilis* plasmid transfer

To further investigate the non-conjugative interspecies plasmid transfer, we decided to test the possibility of *L. lactis* to deliver the genetic material to *B. subtilis*. This time, as a donor strain, we used *L. lactis* MG1363 transformed with pNZ8048 (cm^R) and as a recipient *B. subtilis* 168 Δ*comK* (spec^R). Same co-culturing conditions were applied as in the case of the *B. subtilis* to *L. lactis* matings, and the plasmid transfer efficiency was estimated (Figure 4A). Obtained

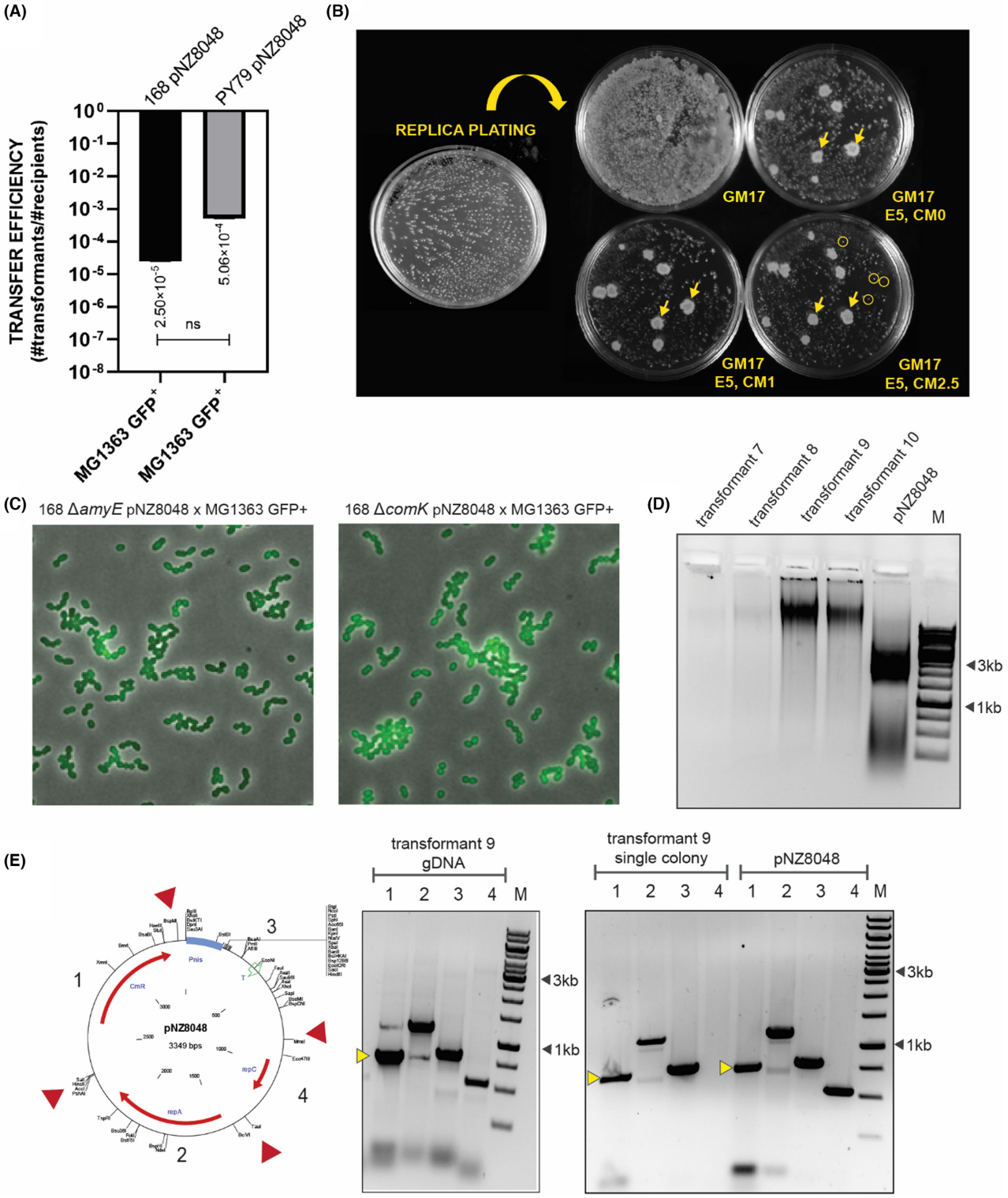

FIGURE 3 pNZ8048 delivery to *L. lactis* MG1363 GFP$^+$ from *B. subtilis*. (A) The average values of pNZ8048 transfer efficiency for *B. subtilis* to *L. lactis* matings. *B. subtilis* 168 and PY79 harbouring pNZ8048 were designed as donor strains and MG1363 with chromosomally integrated and constitutively expressed *sfGFP* as a recipient strain. The statistical analysis was performed in Prism (*t*-test, ns: $p = 0.2961$), the error bars show the standard deviation (SD), $n = 4$. (B) Colony formation after replica plating on GM17 selective medium with reduced concentrations of chloramphenicol. The yellow arrows indicate *B. subtilis* erythromycin resistant colonies. The yellow circles indicate colonies of *L. lactis* transformants picked for further analysis. (C) Micrographs of *L. lactis* MG1363 GFP$^+$ transformants. (D) Gel electrophoresis of isolated plasmids from *L. lactis* transformants, the pNZ8048 lane corresponds to the plasmid isolated from *L. lactis* MG 1363 pNZ8048 strain. (E) PCR analysis of transformant 9. The yellow markers indicate the band representing chloramphenicol cassette.

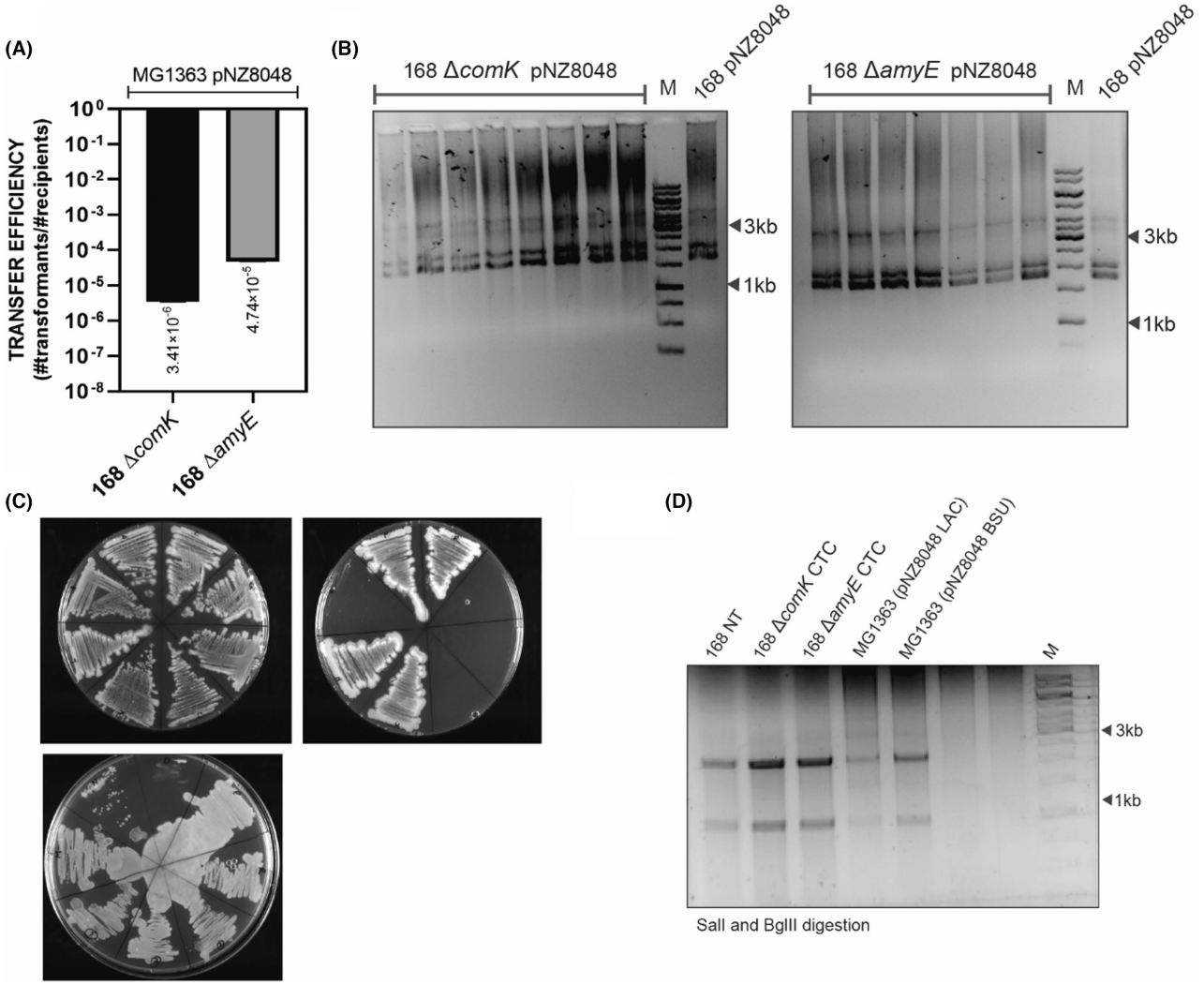

FIGURE 4 *B. subtilis* 168 can acquire pNZ8048 from *L. lactis* MG1363 GFP$^+$. (A) The average values of plasmid transfer efficiency for *L. lactis* to *B. subtilis* matings. The statistical analysis was performed in Prism (*t*-test, ns: $p = 0.3558$), and the error bars show the standard deviation (SD), $n = 5$. (B) Gel electrophoresis of isolated plasmids from *B. subtilis* transformants. (C) Colony formation after replica plating on LB selective medium with chloramphenicol and kanamycin. (D) Restriction analysis of pNZ8048 isolated from different hosts with SalI and BglII restriction enzymes. The analysis of unmodified pNZ8048 should result in two distinct bands 2313 bp and 1036 bp. 168 NT—pNZ8048 was isolated from *B. subtilis* 168 donor strain where it was previously introduced via natural transformation; 168 Δ*comK* CTC and 168 Δ*amyE* CTC—pNZ8048 isolated from the recipient cells that took up the plasmid DNA from *L. lactis* MG1363 pNZ8048 strain; MG1363 (pNZ8048 LAC)—plasmid isolated directly from *L. lactis* MG1363; MG1363 (pNZ8048 BSU)—pNZ8048 was isolated from *B. subtilis* 168 pNZ8048 strain and further introduced to *L. lactis* MG1363 via electroporation.

B. subtilis colonies were replated on selective media with appropriate antibiotics (spectinomycin 100 μg/ml and chloramphenicol 5 μg/ml) and inspected for plasmid presence (Figure 4B,C). Additionally, the integrity of pNZ8048 in donor and recipient strains was confirmed by restriction analysis with SalI and BglII restriction enzymes Figure 4D. We did not observe any changes in the plasmid size that might have occurred upon the transfer from recipient to donor cells. The results demonstrated that competence- negative *B. subtilis* can acquire plasmid DNA from neighbouring *L. lactis* MG1363 cells, and the cell-to-cell plasmid transformation efficiency was estimated on 10^{-6} transformants per CFU recipient.

Presence of the lactococcal sex factor does not facilitate the plasmid transfer between *L. lactis cells*

So far, our data suggest that *L. lactis* can deliver plasmid DNA via cell-to-cell mediated exchange to *B. subtilis*. Next, we proceeded to investigate cell-to-cell transformation between *L. lactis* strains.

L. lactis MG1363 contains a chromosomally located *cluA* gene encoding for sex factor aggregation protein, CluA, involved in conjugative DNA transfer (Godon et al., 1994). To exclude the possibility of plasmid transfer through sex factor mobilization, we transformed *L. lactis* MG1363 with

the pGH9-*cluA* thermosensitive plasmid to markerlessly knock-out *cluA*, resulting in *L. lactis* MG1363 Δ*cluA*. CluA mutant strain was further transformed with pseudo10::Pusp45-sfGFP(Bs) plasmid to obtain *L. lactis* MG1363 GFP$^+$ Δ*cluA* (ery^R) recipient strain. To examine the role of the sex factor in non-conjugative plasmid transfer, we compared the transfer efficiencies for wild type and the *cluA* mutant used separately as a donor and a recipient of pNZ8048. Additionally, we tested transfer of pNZ521 plasmid encoding industrially desired feature—PrtP protease (Gasson, 1983), the presence of which could be tested on casein agar plates.

After the mating procedure on GM17 agar at 30°C and further antibiotic selection with 5 μg/ml erythromycin and 5 μg/ml chloramphenicol, we observed that *L. lactis* can transfer both high copy plasmids, pNZ8048 and pNZ521, with an efficiency of 10^{-3} to 10^{-4} transformants per CFU recipient, respectively (Figure 5A,B). The analysis of the estimated transfer efficiencies also revealed that the presence of the sex factor in MG1363 strain does not facilitate the plasmid transfer under the studied mating conditions, on a rich, growth-promoting media (Figure 5A,B).

Moreover, we compared the plasmid transfer efficiencies to wild type and the *cluA* mutant strain of *L. lactis* MG1363 from the *B. subtilis* PY79 strain and confirmed that a functional *cluA* is not required for the cell-to-cell plasmid transfer under the conditions studied (Figure 5D).

Cell-to-cell plasmid transfer from *S. thermophilus* to *B. subtilis*

Another industrially relevant bacterium, used for milk fermentation is *S. thermophilus*. This Gram-positive bacterium has been reported to be able to induce natural competence. The activation of competence and assembly of the competence machinery is dictated by the ComX pheromone abundance in the environment, whose production and secretion are typically initiated in response to specific environmental factors (Fontaine et al., 2013; Haustenne et al., 2015). Since earlier we observed that *B. subtilis* and *S. thermophilus* could be co-cultured on a lactose supplemented GM17 and form membranous connections, we wondered whether *S. thermophilus* can deliver plasmid DNA to *B. subtilis*

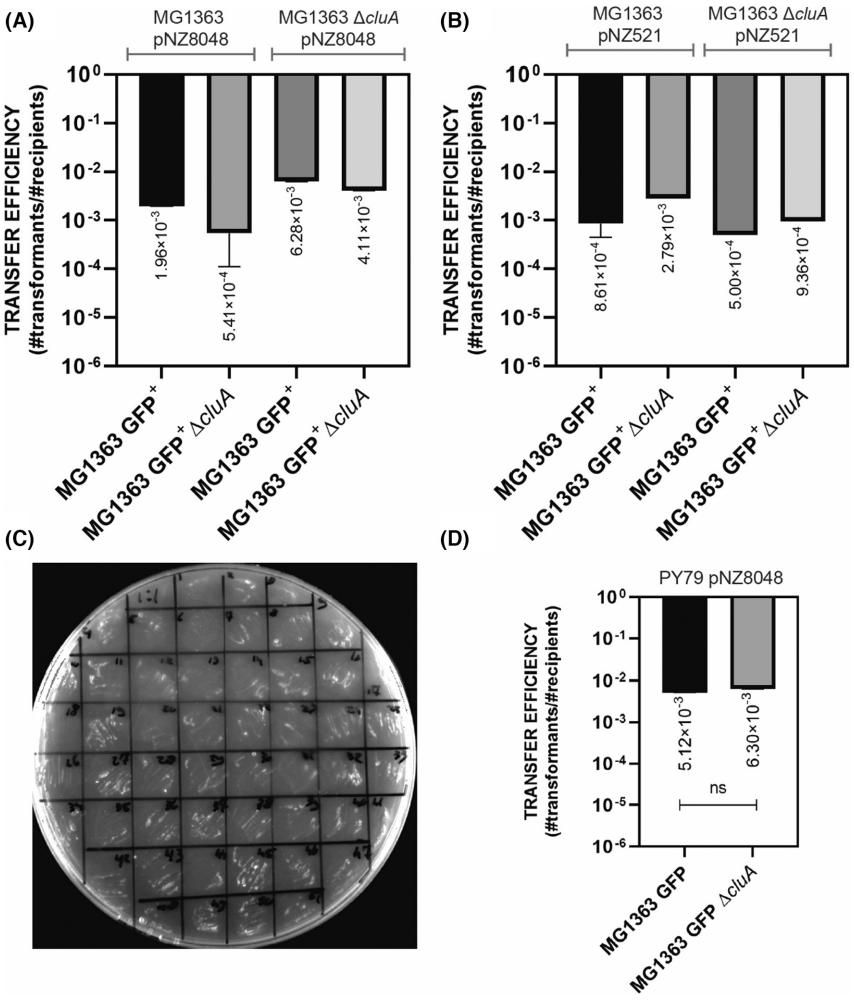

FIGURE 5 Plasmid transfer in conjugation negative strain of *L. lactis* MG1363. (A) The average values of plasmid transfer efficiency for *L. lactis* to *L. lactis* plasmid-transfer matings. The statistical analysis was performed in Prism (Kruskal–Wallis test, ns, not significant), the error bars show the standard deviation (SD), $n = 5$. (B) Transfer efficiency for pNZ521. The statistical analysis was performed in Graph Pad Prism (one-way ANOVA, ns: $p = 0.2770$), and the error bars show the standard deviation (SD), $n = 2$. For both graphs (A) and (B), the names of the donor strains were indicated above the graph, whereas the recipient strains are listed on the *x*-axis. (C) Colony-forming ability of *L. lactis* MG1363 pNZ521 transformants on casein-based medium. (D) Transfer efficiencies for *B. subtilis* to *L. lactis* matings. The error bars show the standard deviation (SD), $n = 5$, *t*-test, ns $p = 0.8718$.

in a cell-to-cell contact manner. Therefore, we tested cell-to-cell transfer of pNZ8048 plasmid from *S. thermophilus* strains, ST-11 and CNRZ302, available in our strain collection, to *B. subtilis* 168 Δ*comK* (spec^R). ST11 and CNRZ302 strains were transformed with pNZ8048 plasmid via electrotransformation and used as donor strains. Additionally, we tested *B. subtilis* 168 Δ*amyE* (tet^R) as a recipient strain. The mating procedure was conducted at 37°C in 1:1 donor to recipient cell ratio on a 1% lactose supplemented GM17 media.

The analysis of the plasmid transfer efficiency showed that *B. subtilis* can efficiently acquire plasmid DNA from *S. thermophilus* strains, similarly to what was observed for *L. lactis* to *B. subtilis* matings. The transfer efficiency for ST-11 strain was estimated on 10^{-6} transformants per CFU recipient, whereas for CNRZ302 10^{-5} transformants per CFU recipient were found. (Figure 6).

DISCUSSION

In this study, we explored the feasibility of intra- and interspecies plasmid transfer, mediated by cell-to-cell interactions in several members of Gram-positive bacteria. First, we confirmed a phenomenon of cell-to-cell mediated HGT in *B. subtilis*, previously described for

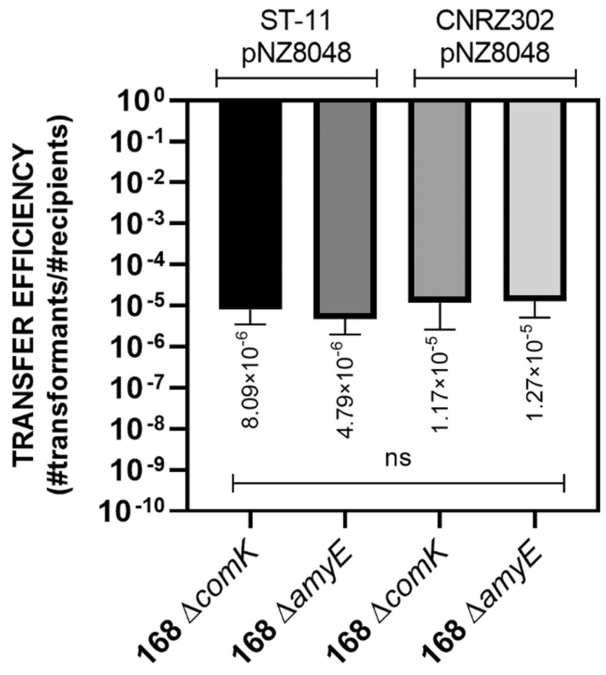

FIGURE 6 Cell-to-cell plasmid transfer from *S. thermophilus* to *B. subtilis*. The graph shows the average of the plasmid transfer efficiencies from ST-11 and CNRZ *S. thermophilus* donor strains (indicated above the graph) to non-competent and competent *B. subtilis* 168 strains, 168 Δ*comK* and 168 Δ*amyE*, respectively (indicated on the *x*-axis). The error bars indicate the SD, *n* = 3. The statistical analysis was performed in Prism (Kruskal–Wallis test, ns, not significant).

mixed strains of *B. subtilis* (Dubey & Ben-Yehuda, 2011; Zhang et al., 2018), and *E. coli* (Maeda et al., 2006; Matsumoto et al., 2016). Second, for the first time, we demonstrated a transfer of a high copy, non-conjugative plasmid in mixed co-cultures of either *B. subtilis* and *L. lactis*, or *B. subtilis* and *S. thermophilus*, showing that cell-to-cell DNA transfer is a ubiquitous form of HGT.

Because *B. subtilis* is known to cause spoilage of dairy products (Arakawa et al., 2008; Faille et al., 2014; Moschonas et al., 2021), we expected it to co-exist with LAB in the glucose or lactose supplemented M17 media. To study the HGT between different species, we tested co-culturing conditions supporting the growth of both donor and recipient strains. We identified the optimal conditions for *B. subtilis*, *L. lactis*, and *S. thermophilus* co-cultures and visualized the single-cell interactions with different microscopy techniques (Figure 1). The membrane staining with the FM5-95 fluorescent dye revealed that these Gram-positive bacteria form membranous structures, previously identified as bacterial nanotubes (Dubey et al., 2016; Dubey & Ben-Yehuda, 2011). Notably, nanotubes have been recently suggested to contribute to the HGT of non-conjugative plasmids in *B. subtilis* on a solid media (Dubey & Ben-Yehuda, 2011). This prompted us to further investigate the non-conjugative cell-to-cell plasmid transfer and explore its boundaries by executing intra-species matings.

Our results show that *B. subtilis* efficiently delivers plasmid DNA to neighbouring *B. subtilis* strains. Since genetic material can be released to the environment through cell lysis or spontaneous DNA secretion by growing cells (Nielsen et al., 2007), it could be argued that obtained transformants acquired the plasmid DNA from the media. By knocking out the gene encoding for the competence master regulator ComK (Hahn et al., 1996; van Sinderen et al., 1994), we excluded the possibility of donor-independent DNA uptake through induction of natural competence for transformation (Figure 2C). Additionally, we demonstrated that plasmid transfer is insensitive to DNase treatment, supporting the results of previous works on cell-to-cell genetic transfer (Dubey & Ben-Yehuda, 2011; Zhang et al., 2018). Resistance to DNase treatment indicates that plasmid DNA is enclosed in a protective conduit, similarly to conjugation-mediated DNA transfer through pili. Yet, here the cell-to-cell plasmid transfer could be distinguished from conjugation by differences in the transfer efficiency of *B. subtilis* 168 strain, harbouring an integrative and conjugative element, ICE*Bs1*, to conjugation-negative *B. subtilis* PY79 (Figure 2C). Notably, the cell-to-cell non-conjugative plasmid transfer between *B. subtilis* strains was more efficient than DNA uptake via transformation (Figure S1A,B). Finally, viable donor cells in the co-culture are essential for mating. We show that recipient cells with and without functional *comK* gene could not take up the plasmid

when added directly to the plates, confirming the requirement of donor and recipient cells to grow in close proximity (Figure 2C). Additionally, when a donor strain was deliberately killed by heat treatment, the mating was unsuccessful (data not shown).

Significantly, our study adds new insight on the mechanisms of HGT in LAB. We demonstrate that plasmid DNA can be transferred by mixed colonies of *B. subtilis* and *L. lactis* (Figures 3 and 4), between *L. lactis* strains (Figure 5), and finally between *B. subtilis* and *S. thermophilus* (Figure 6). Because of several mutations in competence-related genes, *L. lactis* MG1363 is not able to enter the competent state (Mulder et al., 2017). Therefore, for *L. lactis* matings, plasmid transfer did not involve natural transformation. First, we explored the delivery of pNZ8048 (cm^R) from *B. subtilis* to *L. lactis* and showed that *L. lactis* displayed resistance to chloramphenicol after the first round of plating on selective media (Figure 3B). However, the transformants obtained could not grow in liquid media with the same chloramphenicol concentration and grow poorly on a solid selective media after replating. Chloramphenicol acetyltransferase confers resistance to chloramphenicol, and it was found to be transferred between bacteria via nanotubes, showing that acquired chloramphenicol resistance can be a nonhereditary feature (Dubey & Ben-Yehuda, 2011). This would explain the difficulties with the growth of *L. lactis* transformants after replating or culturing in the liquid media with a killing concentration of chloramphenicol (Figure 3B, Figure S2).

However, PCR analysis of *L. lactis* colonies and genomic DNA isolated from the liquid cultures of transformants grown in a lower concentration of chloramphenicol revealed pNZ8048 amplicons (Figure 3E), suggesting that pNZ8048 from *B. subtilis* origin likely resides in the *L. lactis* transformants, perhaps with a reduced copy number. The reduced copy number of pNZ8048 plasmid could result from the differences in the restriction-modification (R/M) systems active in *B. subtilis* and *L. lactis*. *B. subtilis* can recognize 5′ CTCGAG 3′ sequences and methylate with BsuM R/M system, which consists of two operons, BsuMM operon (*ydiO-ydiP*) for two cytosine DNA methyltransferases, and BsuMR operon (*ydiR-ydiS-ydjA*) for a restriction nuclease and two associated proteins of unknown function (Guha, 1988; Maehara et al., 2011; Matsuoka et al., 2005). Notably, pNZ8048 plasmid contains the recognition sequence for the BsuM R/M system, and it is likely modified in the *B. subtilis* host. We suggest that during the cell-to-cell plasmid transfer from *B. subtilis* to *L. lactis* most of the pNZ8048 transferred copies are methylated and could be subsequently recognized by *L. lactis* R/M as foreign DNA (O'sullivan et al., 2000; Schouler et al., 1998). Moreover, the differences in methylation patterns of incoming DNA via natural transformation and host's DNA greatly influence the transformation efficiency (Beauchamp et al., 2017). Our preliminary experiments revealed that pNZ8048 isolated from *B. subtilis* was insensitive to the digestion with an isoschisomer of BsuM, XhoI (Figure S3). This points to pNZ8048 methylation of 5′ CTCGAG 3′ sequence in *B. subtilis*, which protected the plasmid DNA from XhoI activity. Conversely, pNZ8048 isolated from *L. lactis* host could be cleaved with XhoI. Moreover, we transformed pNZ8048 isolated from different hosts to *L. lactis* MG1363 and showed 10-fold reduction in transformation efficiency with pNZ8048 isolated from *B. subtilis* host. However, to fully understand this phenomenon, future studies are needed. We believe that enumeration of pNZ8048 copy numbers in *L. lactis* transformants will shed more light on the effect of methylation in *B. subtilis* donor strain on interspecies transfer of DNA.

Next, we demonstrated that not only *B. subtilis* could transfer plasmids to adjacent *B. subtilis* cells, and the intraspecies cell-to-cell plasmid transfer also occurred between *L. lactis* strains. Additionally, with the *cluA* mutant of *L. lactis* MG1363, we showed that the transfer was conjugation independent and could be applied to deliver industrially-relevant plasmid pNZ521 (Figure 5).

The differences in the average transformation efficiencies in studied bacteria revealed that cell-to-cell mediated plasmid transfer was the most successful for intraspecies matings of *B. subtilis* with *B. subtilis* and *L. lactis* with *L. lactis* (Figure S1B). However, in the case of mixed-species matings with *B. subtilis* as either donor or recipient strain, the efficiency dropped 1000-fold on average. It can be speculated that the differences between intra- and interspecies transfer of the same non-conjugative plasmid might be due to different cell surface and colony-forming properties of *B. subtilis*, *L. lactis*, and *S. thermophilus*, which consequently might affect the formation of stable cell-to-cell connections (Figure S1C). Notably, the most efficient transfer was observed for *B. subtilis*, known for its potent biofilm production abilities (Arnaouteli et al., 2021). Although *L. lactis* is also able to form biofilms (Habimana et al., 2009; Oxaran et al., 2012), they are less abundant than those found in *B. subtilis*, which is reflected in the macrocolony morphology (Figure S1C). As biofilms greatly contribute to the HGT in bacteria (Maeda et al., 2006; Molin & Tolker-Nielsen, 2003), we suggest that the ability to form abundant biofilms by *B. subtilis* is an important feature for enhanced cell-to-cell transfer. Interestingly, the formation of cell-to-cell connections via nanotubes and molecular transfer in *B. subtilis* requires the activity of YmdB (Dubey et al., 2016; Stempler et al., 2017), a phosphodiesterase that affects flagellin expression and biofilm formation (Diethmaier et al., 2014, 2011).

We also showed that within a mixed culture with *S. thermophilus*, *B. subtilis* can acquire plasmid DNA without employing natural competence. These promising

results will be further explored focusing on DNA transfer to *S. thermophilus* from *B. subtilis* and *L. lactis* donor strains.

To conclude, we showed that co-residing bacteria employ HGT in a laboratory setup by establishing a physical contact distinct from the classical conjugation mechanism and does not involve natural transformation. This newly described property of non-conjugative plasmid transfer in LAB is undoubtedly a promising platform for natural strain improvement and should be explored in other industrially-relevant bacteria.

AUTHOR CONTRIBUTIONS

Luiza P. Morawska: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Oscar P. Kuipers:** Conceptualization (equal); funding acquisition (lead); investigation (supporting); methodology (supporting); resources (lead); supervision (lead); validation (lead); writing – original draft (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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