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# Renaissance of traditional DNA transfer strategies for improvement of industrial lactic acid bacteria

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The ever-expanding genomic insight in natural diversity of lactic acid bacteria (LAB) has revived the industrial interest in traditional and natural genetic mobilization methodologies. Here, we review recent advances in horizontal gene transfer processes in LAB, including natural competence, conjugation, and phage transduction. In addition, we envision the possibilities for industrial strain improvement arising from the recent discoveries of molecular exchanges between bacteria through nanotubes and extracellular vesicles, as well as the constantly expanding genome editing possibilities using the CRISPR-Cas technology.

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## Introduction

The lactic acid bacteria (LAB) are of great importance in industrial fermentation and are probably best known for their role in the dairy industry, but certainly also play a key role in a variety of fermentation processes using other food-raw materials and feed-raw materials. Moreover, a continuously expanding panel of LAB strains is marketed as health promoting probiotics. An important industrial innovation strategy is the improvement, expansion and diversification of the starter and probiotic culture repertoire for the reliable production of healthy and tasty consumer products. With the present capacities in microbial genomics, our knowledge of the molecular biology of the LAB is rapidly expanding, providing us with an unprecedented view of the diversity and evolution of these industrially important bacteria and exemplifying the evolutionary importance of horizontal gene transfer and mobile genetic elements (MGEs) [1–3]. At an accelerating rate, we are discovering the core- and pangenomes of a variety of industrially relevant LAB species, including isolates originating from various environments (e.g. plant, intestine, etc.) or artisanal fermentation products. Such isolates often encode phenotypes that are of interest for industrial exploitation, such as stress robustness, flavor formation, bacteriocin production, substrate utilization, and bacteriophage resistance. Although comparative genomics, gene-trait matching and genetic engineering can establish the genetic basis of the relevant phenotypes, it is still a challenge to harness these biodiversity-derived discoveries in industrial strains without applying genetic modification methodologies. This has inspired a renewed interest in naturally occurring horizontal gene transfer processes, including natural competence, phage transduction and conjugation, for the mobilization of traits of interest to industrial strains (Figure 1).

## Natural competence

Natural competence is a cellular state in which bacterial cells are able to internalize exogenous DNA through a dedicated DNA uptake machinery that imports single stranded material. Once intracellular, the single stranded DNA is actively stabilized and subsequently maintained as a plasmid or is incorporated into the chromosome [4]. Among the industrial LAB, natural competence was first established in the yoghurt bacterium *Streptococcus thermophilus* in which formation of the quorum sensing complex ComRS results in expression of the master regulator of competence ComX [5]. The increased ComX level drives the expression of the DNA uptake machinery, a multiprotein complex composed of ComEA, ComEC, ComFA and ComFC, and several secondary competence proteins that facilitate DNA uptake (pilus-like structure proteins ComGA-GG) and protect internalized DNA (RecA, SsbA, SsbB, DprA). This state of natural competence was observed when *S. thermophilus* was grown in chemically defined medium or when synthetic peptides representing the C-terminal of ComS were added [6,7]. It has been used to transfer the gene encoding the extracellular protease PrtP to proteolytically negative strains [8], and to generate histidine prototrophy in strains auxotrophic for this amino acid [9].
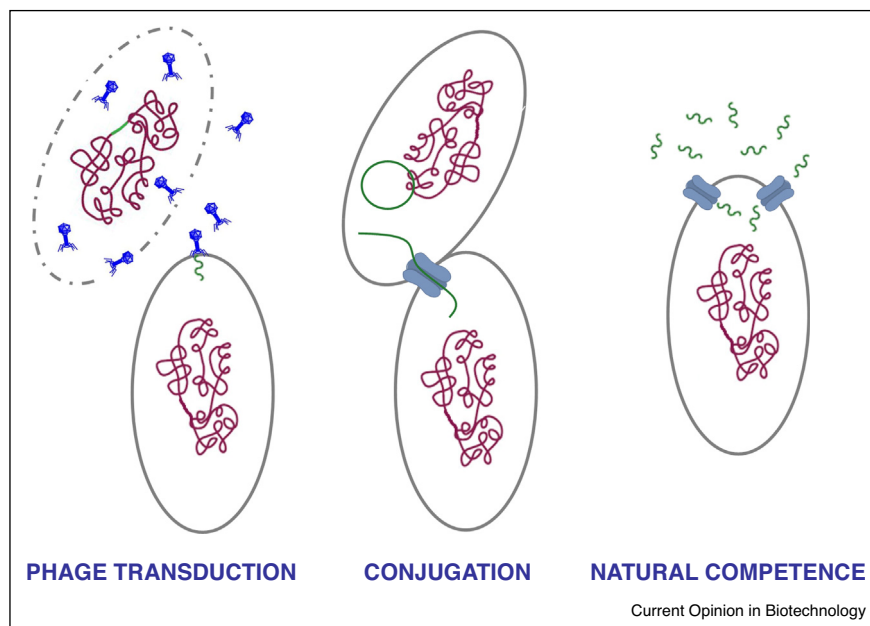
Schematic representation of the 3 'traditional' strategies for genetic mobilization.

Although the presence of (remnants of) the competence genes was observed more than a decade ago in *Lactococcus lactis* [10], it was only recently shown that moderate overexpression of *comX* indeed resulted in the associated capability to internalize DNA [11•,12•]. Similarly, overexpression of an alternative sigma factor led to the induction of competence genes in *Lactobacillus sakei*, although in this organism no transformation could be observed under the conditions tested [13]. To evaluate the phylogenetic conservation of this genotype among the lactobacilli, we evaluated the completeness of the gene set encoding the DNA uptake machinery in subset of *Lactobacillus* genomes (Table 1), and concluded that for all of these species, strains could be identified that encode a complete gene set, although in specific (NCBI-reference) strains one or more of these genes appear to be disrupted by mutations. Although requiring experimental validation, this implies that the natural competence phenotype potentially can be activated in many different LAB, although the regulatory mechanisms underlying competence activation in these bacteria remains to be elucidated. Nevertheless, the broad distribution of the genes required for the DNA uptake machinery may enable novel approaches towards gene-exchange and phenotype-exchange between strains. The acceptance of such strains in the food industry from a regulatory point of view would be tremendously aided by the identification of the natural conditions that trigger the uptake of DNA which are currently only established for specific *S. thermophilus* strains [9].

## Conjugation

Conjugative plasmids as well as integrative and conjugative elements (ICEs) are vertically propagated during replication and cell division. These conjugative MGEs encode similar type IV secretion mobilization machineries that are involved in *oriT*-dependent conjugal transfer to appropriate recipient cells, but also encode distinct functions involved in chromosomal integration and excision (ICEs), and extra-chromosomal replication (plasmids) [14•,15]. The genetically conserved functions of these conjugative MGEs have been exploited in tools aiming to detect them in bacterial genome sequences [16•,17,18], while delimitation of ICEs can be achieved by pan-genome and core-genome mapping [16•] or by curing them from the host chromosome [19•]. Besides their canonical functions, the conjugative MGEs encode a variable number of accessory genes ('cargo') that confer phenotypes to host cells [15,20]. Since their cargo encompasses a number of relevant industrial traits, conjugative MGEs have received considerable attention in LAB. For example, in *L. lactis* genes encoding lactose utilization, extracellular proteinase, and polysaccharide production are commonly encoded on conjugal plasmids [20], whereas nisin production as well as sucrose and raffinose utilization are encoded on ICEs [21,22]. Mobilization of these elements allows the combination of beneficial traits in a single strain [23], or alteration of a strain's capacity to interact with its environment [24]. However, MGEs have also been associated with undesirable traits like antibiotic resistance. This is particularly common among various streptococci, including *S. thermophilus* [25]. On the one

**Table 1**

Natural competence in lactobacilli. The established genes encoding the DNA uptake machinery of *Lactococcus lactis* KF147 [12•] were used to identify homologous genes in *Lactobacillus plantarum* WCFS1, identifying a complete late competence geneset in this species. Subsequently, the *L. plantarum* competence genes were used to search the genomes of a set of other *Lactobacillus* genomes (initially targeting the NCBI-reference genome for each species). When the *Lactobacillus* reference genome sequences contained disrupted competence genes (pseudogenes), it was evaluated whether other genomes of the same species contained intact versions of these genes. Notably, the *comEB* and *comC* genes are known to be absent or not expressed in lactococci that have been experimentally established to be able to become competent, which implies that these genes are not essential for competence development. Taken together the results of this *in silico* analysis indicate that in all *Lactobacillus* species evaluated here there are at least some representative strains that encode a complete geneset for the physical DNA uptake machinery

Protein length (nr. of residues)/protein sequence similarity (%[a]) compared to *L. plantarum* WCFS1 (or *L. lactis* KF147 in the case of *L. plantarum* WCFS1)

| Species | strain | ComC | | ComEA | | ComEB | | ComEC | | ComFA | | ComFC | | ComGA | | ComGB | | ComGC | | ComGD | | ComGE | | ComGF | | ComGG | | ComX | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lactococcus lactis* | KF147 | 221 | NA | 215 | NA | Absent | NA | 736 | NA | 440 | NA | 216 | NA | 312 | NA | 357 | NA | 127 | NA | 143 | NA | 98 | NA | 141 | NA | 94 | NA | 163 | NA |
| *Lactobacillus plantarum* | WCFS1 | 226 | 39 | 241 | 54 | 161 | NA | 763 | 45 | 450 | 51 | 224 | 53 | 324 | 56 | 349 | 47 | 118 | 45 | 157 | 36 | 70 | 38 | 162 | 31 | 54 | 24 | 187 | 42 |

Comparative analysis with other *Lactobacillus* genomes, using the *L. plantarum* WCFS1 protein sequences

| Species | strain | ComC | | ComEA | | ComEB | | ComEC | | ComFA | | ComFC | | ComGA | | ComGB | | ComGC | | ComGD | | ComGE | | ComGF | | ComGG | | ComX | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lactobacillus rhamnosus* | GG | Absent | | 221 | 52 | Absent | | 734 | 47 | 421 | 53 | 223 | 53 | 289 | 55 | 317 | 44 | 106 | 38 | 140 | 39 | 103 | 32 | 155 | 31 | 107 | 24 | 179 | 44 |
| *Lactobacillus acidophilus* | NCFM | 229 | 42 | 227 | 54 | Absent | | 762 | 48 | 427 | 57 | 231 | 48 | 324 | 61 | 334 | 46 | 119 | 54 | 142 | 34 | 89 | 28 | 187 | 37 | 57 | 36 | 178 | 48 |
| *Lactobacillus paracasei* | ATCC 334 182 46 | Absent | | 223 | 51 | Absent | | | | Pseudogene[b] | | 420 | 54 | 222 | 51 | 288 | 56 | 317 | 43 | 107 | 39 | 146 | 38 | 106 | 33 | 153 | 33 | 110 | 29 |
| *Lactobacillus salivarius* | UCC118 | 218 | 47 | 229 | 51 | 158 | 86 | 753 | 51 | 445 | 66 | 228 | 54 | 327 | 64 | 357 | 56 | 100 | 53 | 140 | 37 | 84 | 46 | Pseudogene[b] | | 93 | 22 | 192 | 42 |
| *Lactobacillus fermentum* | IFO 3956 | 241 | 46 | 224 | 53 | 159 | 83 | 745 | 57 | 438 | 67 | 224 | 56 | 319 | 62 | 327 | 49 | 105 | 58 | 161 | 40 | 98 | 34 | 147 | 43 | 79 | 37 | 192 | 45 |
| *Lactobacillus gasseri* | ATCC 33323 | 225 | 46 | 227 | 52 | Absent | | 761 | 48 | 422 | 55 | 223 | 50 | 325 | 62 | 326 | 43 | 98 | 55 | 138 | 38 | 72 | 42 | 172 | 37 | 52 | 35 | 185 | 42 |
| *Lactobacillus helveticus* | CNRZ 32 | 227 | 45 | 231 | 51 | Absent | | 762 | 48 | 428 | 57 | 231 | 48 | 324 | 61 | 333 | 43 | 116 | 51 | 143 | 31 | 89 | 33 | 166 | 38 | 58 | 38 | 181 | 47 |
| *Lactobacillus reuteri DSM* | DSM 20016 | 224 | 48 | 210 | 53 | 161 | 84 | 703 | 51 | 443 | 67 | 226 | 62 | 325 | 67 | 356 | 49 | 103 | 55 | 144 | 45 | 96 | 34 | 143 | 40 | 68 | 38 | 191 | 45 |
| *Lactobacillus sanfranciscensis* | TMW 1.1304 | Pseudogene[b] | | 227 | 53 | 161 | 83 | 742 | 52 | Pseudogene[b] | | 224 | 55 | 324 | 62 | 336 | 50 | 99 | 50 | 148 | 43 | 56 | 42 | 144 | 38 | 55 | 42 | 194 | 41 |

NA = Not applicable.

[a] Needleman-Wunsch Global Align Protein Sequences tool in protein BLAST.

[b] The protein is encoded in the genome of other strains of the same species.

hand, transfer of conjugative MGEs appears to be constrained to an MGE-specific range of compatible acceptor strains [26], whereas on the other hand ICEs have been reported to be transferable across the species border [27]. Intriguingly, it has been proposed that conjugative plasmid and ICE lifestyles of MGEs are inter-changeable and play distinct roles in bacterial evolution, in which plasmids display increased genetic plasticity but have a more constrained host-range than their ICE counterparts [28].

Taken together the conjugative MGEs often encode industrially relevant traits, and genomics combined with dedicated search engines enables the discovery of new conjugative plasmids and ICEs. To better harness their potential in industrial strain improvement approaches it is important to better understand their mechanism of transfer and the cognate host-range limitations. In this context it is also important to better understand the role of group II introns, like the one present in the *L. lactis* sex-factor [29], in the modulation of transfer efficiencies of conjugative MGEs [30].

## Bacteriophage transduction

Bacteriophages are viruses that infect bacterial cells, hijacking the host replication, transcription and translation machineries to drive their proliferation. Bacteriophages infecting LAB have been extensively investigated as they represent one of the major causes of fermentation failure in dairy factories. The majority of phages infecting LAB belong to the *Siphoviridae* family, complemented with members of the *Myoviridae* and *Podoviridae* family, each with distinct phage tail characteristics [31]. For most species within the *Siphoviridae* family, including the species most frequently encountered in the dairy environment (P335, 936 and C2 [32]), panviromes have been established [31,33]. Two main modes of packaging have been recognized, based on either cohesive ends (*cos* phages) or headful packaging (*pac* phages). The latter mode of packaging is initiated on a single recognition sequence and terminated when the phage head is full, a process that is prone to promiscuous packaging of host DNA [31]. Plasmid or chromosomal genes involved in sugar fermentation, proteolysis or antibiotic resistance were transferred between LAB strains via phage transduction. High-frequency plasmid transduction observed in *L. lactis* was explained by the shortening of the original plasmid to a size that exactly fitted the phage head [34]. Infection of a new host by bacteriophages has led to successful transfer of bacterial DNA between strains of poorly genetically accessible organisms such as *Lactobacillus delbrueckii* [35] or even between different LAB species [36]. However, host-specificity is dictated by the combination of phage-encoded receptor binding proteins (RBPs) that associate with the phage base plate and the cell wall polysaccharide and/or proteinaceous receptors on the host surface [37••]. Even within the phage species 936 five RBPs have been identified [38],

showcasing the strong constraints of phage-host recognition that could limit their potential for genomic mobilization. However, this notion is contrasted by the demonstration that plasmid transduction by a certain phage could be exploited for cross-species plasmid transfer between *L. lactis* and *S. thermophilus* [36]. Another technical challenge lies within the fact that one would need to establish appropriate phage transduction protocols for each individual phage to prevent loss of the receptor population due to phage predation.

Despite these advances in our understanding of phage host recognition only limited attention has been given to generalized genome mobilization by promiscuous packaging of the genetic material of the host used for phage-propagation. Identifying effective transducing bacteriophages in LAB could open novel approaches towards genomic exchange between strains, which could be exploited to harness natural diversity for the improvement of industrial starter cultures, particularly if bacteriophages can be identified that display a broad host specificity.

## Perspectives

Besides the revival of traditional methods described above, a few emerging technologies might also have potential to enable natural DNA transfer or could allow dedicated genome editing and engineering in existing industrial strains.

Nanotubes are tubular membranous bridges between cells for which evidence is mounting that they mediate cytoplasmic molecular trade among neighboring cells of the same and different species [39]. For instance, plasmid transfer has been demonstrated from *Bacillus subtilis* to other species including *Staphylococcus aureus* and *Escherichia coli* [40]. Moreover, *B. subtilis* has been shown to inhibit *Bacillus megaterium* growth through the delivery of a tRNase toxin via nanotubes, allowing nutrient extraction from the paralyzed cells [41]. The fact that extracellular membrane vesicles share their membranous nature with the nanotubes may suggest that these communication vehicles also share similar but currently not fully identified machineries involved in their production, with membrane vehicles fusing to and dissociating from nanotubes [39]. Notably, extracellular vesicles of various bacteria have been shown to contain DNA, metabolites and/or proteins and can fuse with both prokaryotic and eukaryotic cells [42,43]. Thereby both nanotubes and extracellular vesicles provide an enormous potential for the natural distribution and exchange of genetic material and cognate phenotypes between bacteria of the same species as well as across the species border [39]. To the best of our knowledge, there have not been reports of nanotubes in LAB to date, and only few reports have identified extracellular vesicles in different pathogenic streptococci [44–46] and some probiotic lactobacilli [47,48]. Therefore, this mechanism of molecular exchange

deserves more attention in the LAB, to evaluate its potential in genome mobilization and genetic exchange.

The role of the CRISPR-Cas system as a bacterial adaptive immune system involved in acquiring resistance against bacteriophages was pioneered in *S. thermophilus* [49] and *E. coli* [50]. Ever since, CRISPR-Cas systems have been discovered in a variety of bacteria, including several LAB [51]. The composite and dynamic nature of the CRISPR array has proven to be an efficient and practical target for the typing and tracking of bacterial strains, including industrial starter cultures and health-promoting probiotic strains [52,53]. Moreover, the role of the system in the acquisition of phage resistance can be effectively employed to expand phage resistance profiles in specific strains [54].

The CRISPR-Cas system was exploited for the construction of a programmable genome editing toolbox, typically employing the *Streptococcus pyogenes* type-II Cas9 endonuclease (SpyCas9) [55]. Cas9 can be targeted to a specific genetic sequence by a complementary short guiding RNA (sgRNA) provided that the sequence is flanked by the protospacer adjacent motif (PAM; NGG for SpyCas9). Recently, phage-assisted evolution allowed the adjustment of the PAM-specificity in SpyCas9 derivatives [56*], enabling the expansion of the sequences that can be targeted. Once guided to its target locus, Cas9 introduces a double strand DNA break in the targeted DNA sequence, which is the foundation of its immunity function that protects bacteria against exogenous DNA [49,50,55]. The CRISPR-Cas9 toolbox has been extensively used in eukaryotes where the double-strand breaks introduced by Cas9 can be repaired by non-homologous end joining (NHEJ), which creates out of frame deletions and insertions (INDELs), leading to gene disruption. Alternatively, these double strand breaks can be repaired by homologous recombination (HR) when a 'repair template' is provided in parallel, allowing highly site-specific mutagenesis [57]. Bacteria commonly lack the NHEJ capacity, and double strand DNA breaks are lethal in most bacteria, which caused the application of Cas9 tools in bacteria to lag behind [19*,55,58]. Actually, the lethality of double strand DNA breaks was exploited in the curing of mobile genetic elements like prophages, plasmids, ICEs and genomic islands from various bacteria, including LAB [19*,59,60]. Bacteria do have an endogenous HR machinery, and the application of Cas9-sgRNA in combination with repair templates has proven to be effective in various bacteria, including several LAB and their phages [61–64]. Moreover, a Cas9 derivative that is catalytically inactivated by point mutations (so-called deadCas9; SpyCas9$^{D10A,H840A}$) has been used in gene silencing in different bacteria, including *L. lactis* [65]. Recently, Cas9-base-editor fusion proteins were reported that instead of introducing a double strand DNA break introduce a specific nucleotide

substitution in the target sequence [66**,67]. This next generation of Cas9 tools will probably accelerate the use of these methods in prokaryotes because they avoid the requirement for a repair template and enable effective genome-editing.

The extreme precision of the Cas9 editing approaches enables the highly effective construction of derivatives that are identical to mutants that emerged spontaneously or were generated by random mutagenesis. Mutants constructed by CRISPR-Cas genetic engineering are indistinguishable from mutants produced by methods acceptable for regulatory bodies, which could, or rather should, change legislation perspectives on the classification of these derivatives as genetically modified organisms to ensure enforceable and non-discriminatory legal guidelines. This opinion has also been expressed by the lactic acid bacteria industrial platform (LABIP) after a dedicated workshop in May 2017 [68].

## Concluding remarks

Although several of the gene mobilization strategies discussed here are considered 'classical' in experimental molecular microbiology, they are receiving renewed attention because of their potential to enable the capitalization of the expansion of our knowledge of the genetic and phenotypic diversity among LAB. The application possibilities of the different mobilization strategies range from generic genomic mobilization by natural competence and generalized bacteriophage transduction, to dedicated mobilization of specific traits associated with conjugative MGEs. The latter category is known to encode a variety of industrially relevant traits and has traditionally been exploited to improve starter cultures, for example the construction of proteolytically active, nisin-resistant and nisin-producing, or polysaccharide producing starter cultures [69]. Contrary to natural competence, which is unrestricted by strain compatibility because it involves import of naked DNA and principally allows the transfer of very large DNA fragments, MGE conjugation and phage transduction are restricted by host-range limitations and enable transfer of fragments up to a certain size (defined by the phage packaging capacity, or the ICE delimitation). Our knowhow of these host range limitations is restricted to relatively few well-established examples [20,37**], and increasing our mechanistic understanding of strain-compatibility in conjugation should help to overcome such specificity-borders. Moreover, it can be anticipated that within the extreme diversity of phage repertoires in nature, there may be environmental phages with a much broader host-range as compared to those that have been studied to date on basis of their detrimental activity in industrial fermentation. Expanding research to typical environmental phages from waste streams may allow the isolation of LAB phages that do not cause any industrial problems, but may be

much more prone to accommodate experimental transduction among a wider variety of strains.

Novel approaches using the emerging potential of membranous connections between bacterial cells (within and across species border), like nanotubes or extracellular vesicles offer exciting possibilities for genetic mobilization, although it remains to be established to what extent these processes are non-selective and can actually be employed for generic mobilization.

Irrespective of the transfer technology employed, selection of the acceptor strain that has incorporated and expresses the desired novel genetic trait remains a challenge. Many of the most interesting industrial traits do not allow phenotypic selection (e.g. flavor formation capacity, specific exopolysaccharide production, etc.), and isolating improved strains-enriched with a non-selectable genotype remains challenging and requires extreme-throughput screening possibilities that may be facilitated by the developments in microfluidics and emulsion technology [70•,71,72]. Alternatively, it may be worth investing in strategies that aim to enrich for the genetic loci that are meant to be transferred prior to their actual transfer, simply to reduce the demand on the throughput of the downstream screening model. Employing (RING-)FISH single-molecule detection strategies [73,74] requires fluorescent labelling of cells which is not compatible with post-selection bacterial growth. Overcoming this technological hurdle deserves further attention, since such methodologies could facilitate high-throughput single-cell-based genetic screening and selection using flow-cytometry and sorting.

Finally, following the continuously expanding application of the CRISPR-Cas technology in eukaryotes, the recently emerging advances in this toolbox have overcome the initial problems of lethality of double-strand chromosomal nicks in bacteria and open tremendous possibilities for fine-grained strain improvement strategies offered by nucleotide-specific genome editing. At present the strains resulting from CRISPR-Cas genome editing would be regarded genetically modified, but the strong arguments of enforceability and non-discrimination favor readjustment of legislation in this area, liberating these strategies from this constraint, possibly with an appropriate case by case evaluation regimen to allow surveillance of the engineered organisms.

## Conflict of interest statement
Nothing declared.

## Acknowledgement

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