University of Groningen

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

Bron, Peter A; Marcelli, Barbara; Mulder, Joyce; van der Els, Simon; Morawska, Luiza P; Kuipers, Oscar P; Kok, Jan; Kleerebezem, Michiel

Published in:
Current Opinion in Biotechnology

DOI:
[10.1016/j.copbio.2018.09.004](https://doi.org/10.1016/j.copbio.2018.09.004)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Bron, P. A., Marcelli, B., Mulder, J., van der Els, S., Morawska, L. P., Kuipers, O. P., Kok, J., & Kleerebezem, M. (2019). Renaissance of traditional DNA transfer strategies for improvement of industrial lactic acid bacteria. *Current Opinion in Biotechnology*, 56, 61-68.
<https://doi.org/10.1016/j.copbio.2018.09.004>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

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

Peter A Bron^{1,2}, Barbara Marcelli^{1,3}, Joyce Mulder^{1,3},
Simon van der Els^{1,4}, Luiza P Morawska^{1,3}, Oscar P Kuipers^{1,3},
Jan Kok^{1,3} and Michiel Kleerebezem^{1,4}

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.

Addresses

¹ BE-Basic, Delft, The Netherlands

² NIZO, Ede, The Netherlands

³ RUG, Groningen, The Netherlands

⁴ Host Microbe Interactomics Group, Wageningen University, Wageningen, The Netherlands

Corresponding author: Bron, Peter A (peter.bron@nizo.com)

Current Opinion in Biotechnology 2019, 56C:61–68

This review comes from a themed issue on Food biotechnology

Edited by Rute Neves and Herwig Bachmann

<https://doi.org/10.1016/j.copbio.2018.09.004>

0958-1669/© 2018 Elsevier Ltd. All rights reserved.

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].

Figure 1

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^a] 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
<i>Lactococcus lactis</i>	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
<i>Lactobacillus plantarum</i>	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 <i>Lactobacillus</i> genomes, using the <i>L. plantarum</i> WCFS1 protein sequences															
<i>Lactobacillus rhamnosus</i>	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
<i>Lactobacillus acidophilus</i>	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
<i>Lactobacillus paracasei</i> 182	ATCC 334 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
<i>Lactobacillus salivarius</i>	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
<i>Lactobacillus fermentum</i>	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
<i>Lactobacillus gasseri</i>	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
<i>Lactobacillus helveticus</i>	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
<i>Lactobacillus reuteri</i> 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
<i>Lactobacillus sanfranciscensis</i>	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 vesicles 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

This work was carried out within the BE-Basic R&D Program, which was granted an FES subsidy from the Dutch Ministry of Economic Affairs.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Wu C, Huang J, Zhou R: **Genomics of lactic acid bacteria: current status and potential applications.** *Crit Rev Microbiol* 2017, **43**:393-404.
2. Stefanovic E, Fitzgerald G, McAuliffe O: **Advances in the genomics and metabolomics of dairy lactobacilli: a review.** *Food Microbiol* 2017, **61**:33-49.
3. Sun Z, Harris HM, McCann A, Guo C, Argimon S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF *et al.*: **Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera.** *Nat Commun* 2015, **6**:8322.
4. Blokesch M: **Natural competence for transformation.** *Curr Biol* 2016, **26**:R1126-R1130.
5. Fontaine L, Boutry C, de Frahan MH, Delplace B, Fremaux C, Horvath P, Boyaval P, Hols P: **A novel pheromone quorum-sensing system controls the development of natural competence in *Streptococcus thermophilus* and *Streptococcus salivarius*.** *J Bacteriol* 2010, **192**:1444-1454.
6. Blomqvist T, Steinmoen H, Havarstein LS: **Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*.** *Appl Environ Microbiol* 2006, **72**:6751-6756.
7. Gardan R, Besset C, Guillot A, Gitton C, Monnet V: **The oligopeptide transport system is essential for the development of natural competence in *Streptococcus thermophilus* strain LMD-9.** *J Bacteriol* 2009, **191**:4647-4655.
8. Dandoy D, Fremaux C, de Frahan MH, Horvath P, Boyaval P, Hols P, Fontaine L: **The fast milk acidifying phenotype of *Streptococcus thermophilus* can be acquired by natural transformation of the genomic island encoding the cell-envelope proteinase PrtS.** *Microb Cell Fact* 2011, **10**(Suppl. 1): S21.
9. Fontaine L, Dandoy D, Boutry C, Delplace B, de Frahan MH, Fremaux C, Horvath P, Boyaval P, Hols P: **Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in *Streptococcus thermophilus*.** *Appl Environ Microbiol* 2010, **76**:7870-7877.
10. Wydau S, Dervyn R, Anba J, Dusko Ehrlich S, Maguin E: **Conservation of key elements of natural competence in *Lactococcus lactis* ssp.** *FEMS Microbiol Lett* 2006, **257**:32-42.
11. David B, Radziejewski A, Toussaint F, Fontaine L, Henry de Frahan M, Patout C, van Dillen S, Boyaval P, Horvath P, Fremaux C *et al.*: **Natural DNA transformation is functional in *Lactococcus lactis* ssp. *cremoris* KW2.** *Appl Environ Microbiol* 2017, **83**: e01074-01017.
12. Mulder J, Wels M, Kuipers OP, Kleerebezem M, Bron PA: **Unleashing natural competence in *Lactococcus lactis* by induction of the competence regulator ComX.** *Appl Environ Microbiol* 2017, **83**:e01320-01317.
13. Schmid S, Bevilacqua C, Crutz-Le Coq A-M: **Alternative sigma factor σ^H activates competence gene expression in *Lactobacillus sakei*.** *BMC Microbiol* 2012, **12**:32.
14. Cury J, Oliveira PH, de la Cruz F, Rocha EPC: **Host range and genetic plasticity explain the co-existence of integrative and extrachromosomal mobile genetic elements.** *Mol Biol Evol* 2018, **35**:2230-2239.

This paper proposes that conjugative plasmids and ICE lifestyles are interchangeable and play distinct roles in bacterial evolution, that is having increased genetic plasticity and expanded host range, respectively.

15. Johnson CM, Grossman AD: **Integrative and conjugative elements (ICEs): what they do and how they work.** *Annu Rev Genet* 2015, **49**:577-601.
16. Cury J, Touchon M, Rocha EPC: **Integrative and conjugative elements and their hosts: composition, distribution and organization.** *Nucleic Acids Res* 2017, **45**:8943-8956.
- This paper describes an elegant method for the delimitation of ICEs by exploiting the principal absence of core genome functions in ICEs.
17. Li X, Xie Y, Liu M, Tai C, Sun J, Deng Z, Ou HY: **oriTfinder: a web-based tool for the identification of origin of transfers in DNA sequences of bacterial mobile genetic elements.** *Nucleic Acids Res* 2018, **46**(W1):W229-W234.
18. Oliveira Alvarenga D, Moreira LM, Chandler M, Varani AM: **A practical guide for comparative genomics of mobile genetic elements in prokaryotic genomes.** *Methods Mol Biol* 2018, **1704**:213-242.
19. van der Els S, James JK, Kleerebezem M, Bron PA: **Versatile Cas9-driven subpopulation selection toolbox for *Lactococcus lactis*.** *Appl Environ Microbiol* 2018, **84**:e02752-02717.
- This paper describes a Cas9-based method for effective curing and effective delimitation of mobile genetic elements.
20. Ainsworth S, Stockdale S, Bottacini F, Mahony J, van Sinderen D: **The *Lactococcus lactis* plasmidome: much learnt, yet still lots to discover.** *FEMS Microbiol Rev* 2014, **38**:1066-1088.
21. De Vos WM, Beerthuyzen MM, Luesink EL, Kuipers OP: **Genetics of the nisin operon and the sucrose-nisin conjugative transposon Tn5276.** *Dev Biol Stand* 1995, **85**:617-625.
22. Machielsens R, Siezen RJ, van Hijum SA, van Hylckama Vlieg JE: **Molecular description and industrial potential of Tn6098 conjugative transfer conferring alpha-galactoside metabolism in *Lactococcus lactis*.** *Appl Environ Microbiol* 2011, **77**:555-563.
23. Mills S, Griffin C, O'Connor PM, Serrano LM, Meijer WC, Hill C, Ross RP: **A multibacteriocin cheese starter system, comprising nisin and lactacin 3147 in *Lactococcus lactis*, in combination with Plantaricin from *Lactobacillus plantarum*.** *Appl Environ Microbiol* 2017, **83**.
24. Tarazanova M, Beerthuyzen M, Siezen R, Fernandez-Gutierrez MM, de Jong A, van der Meulen S, Kok J, Bachmann H: **Plasmid complement of *Lactococcus lactis* NCDO712 reveals a novel pilus gene cluster.** *PLoS One* 2016, **11**:e0167970.
25. Florez AB, Mayo B: **Antibiotic resistance-susceptibility profiles of *Streptococcus thermophilus* isolated from raw milk and genome analysis of the genetic basis of acquired resistances.** *Front Microbiol* 2017, **8**:2608.
26. Dahmane N, Robert E, Deschamps J, Meylheuc T, Delorme C, Briandet R, Leblond-Bourget N, Guedon E, Payot S: **Impact of cell surface molecules on conjugative transfer of the integrative and conjugative element ICES₃ of *Streptococcus thermophilus*.** *Appl Environ Microbiol* 2018, **84**:e02109-02117.
27. Dahmane N, Libante V, Charron-Bourgoin F, Guedon E, Guedon G, Leblond-Bourget N, Payot S: **Diversity of integrative and conjugative elements of *Streptococcus salivarius* and their intra- and interspecies transfer.** *Appl Environ Microbiol* 2017, **83**:e00337-00317.
28. Cury J, Oliveira PH, de la Cruz F, Rocha EPC: **Host range and genetic plasticity explain the co-existence of integrative and extrachromosomal mobile genetic elements.** *Mol Biol Evol* 2018, **35**:2230-2239.
29. Novikova O, Smith D, Hahn I, Beauregard A, Belfort M: **Interaction between conjugative and retrotransposable elements in horizontal gene transfer.** *PLoS Genet* 2014, **10**:e1004853.
30. Qu G, Piazza CL, Smith D, Belfort M: **Group II intron inhibits conjugative relaxase expression in bacteria by mRNA targeting.** *eLife* 2018, **7**:e34268.
31. Mahony J, Bottacini F, van Sinderen D, Fitzgerald GF: **Progress in lactic acid bacterial phage research.** *Microb Cell Fact* 2014, **13** (Suppl. 1):S1.
32. Deveau H, Labrie SJ, Chopin MC, Moineau S: **Biodiversity and classification of lactococcal phages.** *Appl Environ Microbiol* 2006, **72**:4338-4346.
33. Ventura M, Canchaya C, Bernini V, Altermann E, Barrangou R, McGrath S, Claesson MJ, Li Y, Leahy S, Walker CD et al.: **Comparative genomics and transcriptional analysis of prophages identified in the genomes of *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus casei*.** *Appl Environ Microbiol* 2006, **72**:3130-3146.
34. Wegmann U, Overweg K, Jeanson S, Gasson M, Shearman C: **Molecular characterization and structural instability of the industrially important composite metabolic plasmid pLP712.** *Microbiology* 2012, **158**:2936-2945.
35. Ravin V, Sasaki T, Raisanen L, Riipinen KA, Alattosava T: **Effective plasmid pX3 transduction in *Lactobacillus delbrueckii* by bacteriophage LL-H.** *Plasmid* 2006, **55**:184-193.
36. Ammann A, Neve H, Geis A, Heller KJ: **Plasmid transfer via transduction from *Streptococcus thermophilus* to *Lactococcus lactis*.** *J Bacteriol* 2008, **190**:3083-3087.
37. Mahony J, Cambillau C, van Sinderen D: **Host recognition by lactic acid bacterial phages.** *FEMS Microbiol Rev* 2017, **41**:S16-S26.
- Excellent review of the state of the art of our understanding of the molecular mechanism of phage-host recognition and its specificity in lactic acid bacteria.
38. Murphy J, Royer B, Mahony J, Hoyles L, Heller K, Neve H, Bonestroo M, Nauta A, van Sinderen D: **Biodiversity of lactococcal bacteriophages isolated from 3 Gouda-type cheese-producing plants.** *J Dairy Sci* 2013, **96**:4945-4957.
39. Baidya AK, Bhattacharya S, Dubey GP, Mamou G, Ben-Yehuda S: **Bacterial nanotubes: a conduit for intercellular molecular trade.** *Curr Opin Microbiol* 2018, **42**:1-6.
40. Dubey GP, Ben-Yehuda S: **Intercellular nanotubes mediate bacterial communication.** *Cell* 2011, **144**:590-600.
41. Stempler O, Baidya AK, Bhattacharya S, Malli Mohan GB, Tzipilevich E, Sinai L, Mamou G, Ben-Yehuda S: **Interspecies nutrient extraction and toxin delivery between bacteria.** *Nat Commun* 2017, **8**:315.
42. Mashburn-Warren LM, Whiteley M: **Special delivery: vesicle trafficking in prokaryotes.** *Mol Microbiol* 2006, **61**:839-846.
43. Schertzner JW, Whiteley M: **Bacterial outer membrane vesicles in trafficking, communication and the host-pathogen interaction.** *J Mol Microbiol Biotechnol* 2013, **23**:118-130.
44. Jhelum H, Sori H, Sehgal D: **A novel extracellular vesicle-associated endodeoxyribonuclease helps *Streptococcus pneumoniae* evade neutrophil extracellular traps and is required for full virulence.** *Sci Rep* 2018, **8**:7985.
45. Uhlmann J, Rohde M, Siemens N, Kreikemeyer B, Bergman P, Johansson L, Norrby-Teglund A: **LL-37 triggers formation of *Streptococcus pyogenes* extracellular vesicle-like structures with immune stimulatory properties.** *J Innate Immun* 2016, **8**:243-257.
46. Haas B, Grenier D: **Isolation, characterization and biological properties of membrane vesicles produced by the swine pathogen *Streptococcus suis*.** *PLoS One* 2015, **10**:e0130528.
47. Behzadi E, Mahmoodzadeh Hosseini H, Imani Fooladi AA: **The inhibitory impacts of *Lactobacillus rhamnosus* GG-derived extracellular vesicles on the growth of hepatic cancer cells.** *Microb Pathog* 2017, **110**:1-6.
48. Li M, Lee K, Hsu M, Nau G, Mylonakis E, Ramratnam B: ***Lactobacillus*-derived extracellular vesicles enhance host immune responses against vancomycin-resistant enterococci.** *BMC Microbiol* 2017, **17**:66.
49. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: **CRISPR provides acquired resistance against viruses in prokaryotes.** *Science* 2007, **315**:1709-1712.
50. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der

- Oost J: **Small CRISPR RNAs guide antiviral defense in prokaryotes.** *Science* 2008, **321**:960-964.
51. Koonin EV, Makarova KS, Wolf YI: **Evolutionary genomics of defense systems in archaea and bacteria.** *Annu Rev Microbiol* 2017, **71**:233-261.
 52. Barrangou R, Dudley EG: **CRISPR-based typing and next-generation tracking technologies.** *Annu Rev Food Sci Technol* 2016, **7**:395-411.
 53. Horvath P, Romero DA, Coute-Monvoisin AC, Richards M, Deveau H, Moineau S, Boyaval P, Fremaux C, Barrangou R: **Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*.** *J Bacteriol* 2008, **190**:1401-1412.
 54. Levin BR, Moineau S, Bushman M, Barrangou R: **The population and evolutionary dynamics of phage and bacteria with CRISPR-mediated immunity.** *PLoS Genet* 2013, **9**:e1003312.
 55. Mougiakos I, Bosma EF, de Vos WM, van Kranenburg R, van der Oost J: **Next generation prokaryotic engineering: the CRISPR-cas toolkit.** *Trends Biotechnol* 2016, **34**:575-587.
 56. Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z *et al.*: **Evolved Cas9 variants with broad PAM compatibility and high DNA specificity.** *Nature* 2018, **556**:57-63.
- This paper describes the phage-assisted evolution of Cas9 to accommodate a broader spectrum of PAM target sequence.
57. Wang H, La Russa M, Qi LS: **CRISPR/Cas9 in genome editing and beyond.** *Annu Rev Biochem* 2016, **85**:227-264.
 58. Cui L, Bikard D: **Consequences of Cas9 cleavage in the chromosome of *Escherichia coli*.** *Nucleic Acids Res* 2016, **44**:4243-4251.
 59. Selle K, Klaenhammer TR, Barrangou R: **CRISPR-based screening of genomic island excision events in bacteria.** *Proc Natl Acad Sci U S A* 2015, **112**:8076-8081.
 60. Jang YJ, Seo SO, Kim SA, Li L, Kim TJ, Kim SC, Jin YS, Han NS: **Elimination of the cryptic plasmid in *Leuconostoc citreum* by CRISPR/Cas9 system.** *J Biotechnol* 2017, **251**:151-155.
 61. Lemay ML, Tremblay DM, Moineau S: **Genome engineering of virulent lactococcal phages using CRISPR-Cas9.** *ACS Synth Biol* 2017, **6**:1351-1358.
 62. Martel B, Moineau S: **CRISPR-Cas: an efficient tool for genome engineering of virulent bacteriophages.** *Nucleic Acids Res* 2014, **42**:9504-9513.
 63. Oh JH, van Pijkeren JP: **CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*.** *Nucleic Acids Res* 2014, **42**:e131.
 64. Song X, Huang H, Xiong Z, Ai L, Yang S: **CRISPR-Cas9(D10A) nickase-assisted genome editing in *Lactobacillus casei*.** *Appl Environ Microbiol* 2017, **83**:e01259-01217.
 65. Berlec A, Skrllec K, Kocjan J, Olenic M, Strukelj B: **Single plasmid systems for inducible dual protein expression and for CRISPR-Cas9/CRISPRi gene regulation in lactic acid bacterium *Lactococcus lactis*.** *Sci Rep* 2018, **8**:1009.
 66. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR: **Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage.** *Nature* 2017, **551**:464-471.
- This paper describes the use of dCas9 fused to base editor functions, enabling targeted, single-step, 'true' genome editing in prokaryotic genomes.
67. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR: **Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage.** *Nature* 2016, **533**:420-424.
 68. Johansen E: **Future access and improvement of industrial lactic acid bacteria cultures.** *Microb Cell Fact* 2017, **16**:230.
 69. Ross RP, Morgan S, Hill C: **Preservation and fermentation: past, present and future.** *Int J Food Microbiol* 2002, **79**:3-16.
 70. Vervoort Y, Linares AG, Roncoroni M, Liu C, Steensels J, Verstrepen KJ: **High-throughput system-wide engineering and screening for microbial biotechnology.** *Curr Opin Biotechnol* 2017, **46**:120-125.
- This paper reviews state of the art high throughput screening methods for microbes, including the application of nano reactors for single cell screening that paves the way to recognize and isolate variants with non-selectable phenotypes.
71. Bachmann H, Fischlechner M, Rabbers I, Barfa N, Branco dos Santos F, Molenaar D, Teusink B: **Availability of public goods shapes the evolution of competing metabolic strategies.** *Proc Natl Acad Sci U S A* 2013, **110**:14302-14307.
 72. Zhu XD, Chu J, Wang YH: **Advances in microfluidics applied to single cell operation.** *Biotechnol J* 2018, **13**.
 73. Zwirgmaier K, Ludwig W, Schleifer KH: **Recognition of individual genes in a single bacterial cell by fluorescence in situ hybridization—RING-FISH.** *Mol Microbiol* 2004, **51**:89-96.
 74. Cerqueira L, Azevedo NF, Almeida C, Jardim T, Keevil CW, Vieira MJ: **DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH).** *Int J Mol Sci* 2008, **9**:1944-1960.