Elsevier Editorial System(tm) for Plasmid
Manuscript Draft

Manuscript Number:

Title: The phage integrase vector pIPI03 allows recA-independent, site-specific labeling of *Staphylococcus lugdunensis* strains

Article Type: Full Article

Keywords: *Staphylococcus*
Vector
Bacteriophage
competitive index
Integration

Corresponding Author: Dr. Tim Foster,

Corresponding Author's Institution: Trinity College Dublin

First Author: Simon Heilbronner, MSc

Order of Authors: Simon Heilbronner, MSc; Ian R Monk, PhD; Tim Foster

Abstract: Abstract

Staphylococcus lugdunensis is a coagulase negative staphylococcus that is a commensal of man and an opportunistic pathogen. A site-specific integrative plasmid for the use in *S. lugdunensis* was constructed and validated. The integrase gene *ccrB* of bacteriophage ϕ SL01 together with its attachment site was cloned into the thermosensitive plasmid pIMAY. The resulting plasmid pIPI03 integrated recA-independently, site-specifically and irreversibly into the *S. lugdunensis* chromosome. Two IPTG-inducible antibiotic resistance determinants were cloned into pIPI03 and the derivatives were used to construct strains suitable for competitive growth experiments in both in vitro and in vivo.

Suggested Reviewers: Tarok Bae PhD
Associate Professor, University of Indiana
tbae@iun.edu

Constructed widely-used plasmid for allelic exchange in staphylococci

Chia Lee PhD
Professor, University of Arkansas
cle2@uams.edu

Wide experience of genetic manipulation of staphylococci Developed vectors for integration at phage attachment sites in *S. aureus*

Francois Vandenesch MD
Professor, University of Lyon
denesch@univ-lyon1.fr

Published important paper on the virulence factors of *S. lugdunensis*. The species was discovered in this institute in the 1980s and its importance was first noted here. Performed some early genetic studies

Soeren Gatermann MD

Professor

soeren.gatermann@rub.de

Has studied pathogenesis of several coagulase negative staphylococci including *S.lugdunensis*.

Performed some of the first genetic manipulations in this species

Andreas Peschel PhD

Professor, University of Tübingen

andreas.peschel@uni-tuebingen.de

Wide experience of molecular pathogenesis of staphylococcal infections.

Opposed Reviewers:

TFoster-528

Your password is: foster844656

Staphylococcus lugdunensis is a commensal and occasional pathogen of humans. Until recently easy genetic manipulation of this organism had not been reported. We previously set up a genetic system involving electrotransformation of plasmids and site specific allelic exchange mutagenesis. In this paper we report the construction and validation of a temperature sensitive plasmid which integrates site-specifically into the chromosome of most strains of *S.lugdunensis*. The vector was modified to allow it to be used to mark strains for competitive growth experiments. This approach could be adapted to other species of *staphylococcus*

Highlights

- Plasmid vector constructed and validate that integrates site-specifically into the chromosome of most strains of *Staphylococcus lugdunensis*.
- Integrates at a phage attachment site directed by a phage integrase
- Vector stably integrated and maintained at 37 °C. No evidence for excision
- Vector labelled with different inducible antibiotic resistance markers
- Used for testing relative fitness of constructs in vitro

1   The phage integrase vector pPI03 allows *recA*-independent, site-specific labeling of
2   *Staphylococcus lugdunensis* strains

3

4

5

6

7

8

9   Simon Heilbronner¹, Ian Monk¹ and Timothy Foster¹

10

11

12   ¹ Microbiology Department, Trinity College, Dublin 2, Ireland.

13

14

15   Running title. The *Staphylococcus lugdunensis* phage-integrase vector pPI03

16

17   Corresponding author. tfoster@tcd.ie

18

19

20

21

Abstract

Staphylococcus lugdunensis is a coagulase negative staphylococcus that is a commensal of man and an opportunistic pathogen. A site-specific integrative plasmid for the use in *S. lugdunensis* was constructed and validated. The integrase gene *ccrB* of bacteriophage ϕ SL01 together with its attachment site was cloned into the thermosensitive plasmid pIMAY. The resulting plasmid pIPI03 integrated *recA*-independently, site-specifically and irreversibly into the *S. lugdunensis* chromosome. Two IPTG-inducible antibiotic resistance determinants were cloned into pIPI03 and the derivatives were used to construct strains suitable for competitive growth experiments in both *in vitro* and *in vivo*.

Keywords

Staphylococcus lugdunensis, plasmid vector, *recA* mutant, bacteriophage

1. Introduction

S. lugdunensis (Freney et al., 1988) is a coagulase negative staphylococcus (CoNS) that shows an unusual degree of virulence. The species is a commensal of human skin that is predominantly isolated of the moist areas such as perineum and the inguinal fold (Bieber and Kahlmeter, 2010). However it is particularly associated with cases of infective endocarditis (Anguera et al., 2005; Patil et al., 2011; Vandenesch et al., 1993) with a course of disease akin to *S. aureus* infections. Although *S. lugdunensis* is recognized to be an important pathogen, remarkably little is known about virulence factors and the molecular mechanisms of pathogenicity. Furthermore, genetic tools for the manipulation of the organism were limited until very recently when efficient transformation by electroporation was reported along with the use of the thermosensitive vector pIMAY for constructing site-specific mutations by allelic exchange (Heilbronner et al., 2013).

Bacteriophage integrases recognize attachment sites (*attB*) on the bacterial chromosome and mediate site-specific recombination with the phage *attP* site. This results in a single copy of the circular phage genome being inserted into the bacterial chromosome (Hirano et al., 2011). It has been demonstrated before that phage integrases can be used to promote site-specific integration into the bacterial chromosome of plasmids harboring an *attP* site. Such integrative plasmids have been used both in Gram-negative and Gram-positive bacterial species including *Lactococcus lactis* (Petersen et al., 2013) *Staphylococcus aureus* (Lei et al., 2012) and *Listeria monocytogenes* (Monk et al., 2008a; Monk et al., 2008b).

We examined the genome sequence of *S. lugdunensis* N920143 (Heilbronner et al., 2011) and identified a prophage ϕ SL1 that is not present in strain HKU09-01 (Tse et al., 2010). The phage integrase of ϕ SL1 was used to construct the integrative plasmid pIPI03 and its derivatives pIPI_{ery} and pIPI_{kan} which were tested in competitive growth experiments.

2. Materials and Methods

Unless stated otherwise, reagents and chemicals were obtained from Sigma Aldrich. Restriction endonucleases were obtained from Roche and Fermentas.

2.1 Bacterial strains and growth conditions

All strains are listed in Table 1. *S. lugdunensis* was grown in tryptic soy broth (TSB) or agar (TSA) (Difco). *E. coli* strains were grown in Luria-Bertani broth (LB) or agar (LA) (Difco). Unless stated otherwise, strains were grown at 37 °C.

2.2 Construction of pIPI03

To construct the *S. lugdunensis* phage integrase vector, the thermosensitive vector pIMAY (Monk et al., 2012) was modified using the SLIC cloning method reported earlier (Li and Elledge,

2007; Li and Elledge, 2012). Amplification of the ϕ SL1 integrase (*ccrB*) together with the corresponding attachment site *attP* was only possible from the circular phage genome and not from the linearized, integrated genome of the lysogenic phage. Thus, *S. lugdunensis* N920143 was grown to exponential phase and induced for 1 h with 1 μ g / ml mitomycin C to stimulate the excision of ϕ SL1. Cells were harvested and DNA was extracted as described previously (Heilbronner et al., 2011). At their 5' ends the primers *ccrB*-F / *ccrB*-R contain 27 bp sequence identity to the pIMAY sequence downstream of the *cat* gene to allow SLIC assembly of vector and insert. The 5.7 kb backbone of pIMAY was amplified (linearized after the *cat* stop codon) using primers pIMAY_SLICcat_F and pIMAY_SLICcat_R. All PCR reactions were carried out using Phusion polymerase (Finnzymes). Primer sequences are summarized in Table 2.

The PCR products were purified and the vector product was digested with DpnI in order to remove the methylated template DNA. 1 μ g of vector and insert DNA was treated with T4 DNA polymerase in a final volume of 40 μ l (creation of single stranded 5' ends). The reaction contained NEB Buffer 2 (New England Biolabs), molecular grade BSA, 5mM DTT, 200 mM Urea and 3 Units T4 DNA polymerase (New England Biolabs). The reaction was incubated for 20 min on 23 °C and stopped by the addition of 25 mM EDTA (pH 8) and subsequent incubation for 20 min at 75 °C. 5 μ l of vector and 5 μ l of insert were mixed and the single stranded overhangs (identical ends generated by the primers *ccrB*-F/*ccrB*-R) were allowed to anneal. For this, the reaction was placed in a PCR machine for 10 min at 65 °C followed by a decrease in temperature from 65 – 25 °C with 1 min hold for each degree. 2.5 μ l of the reaction was subsequently used for the transformation of *E. coli*. Recovered clones were validated by DNA sequencing.

IPTG-inducible kanamycin and erythromycin resistance cassettes (Monk et al., 2008a) were excised from pIMCkan and pIMCery, respectively, using the restriction endonucleases KpnI / SacI. The fragments were gel-purified and cloned into the MCS of pPI03 treated with the same endonucleases. The resulting plasmids were named pPI03kan and pPI03ery.

2.3 Chromosomal integration of pPI03

pPI03 was used to transform *S. lugdunensis* HKU09-01 strains (Heilbronner et al., 2013; Zapotoczna et al., 2012). Due to the thermosensitive character of the plasmid transformants were selected at 28 °C on TSA containing 10 µg / ml chloramphenicol (TSA_{cm10}). To select for plasmid integration, one colony from a plate grown at 28°C (with an autonomously replicating plasmid) was resuspended in TSB and dilutions were plated on TSA_{cm10} and incubated at 37°C. Colonies were screened by PCR to confirm site-specific integration.

To determine the frequency of pPI03 integration, a HKU09-01 (pPI03) colony was resuspended in TSB. Dilutions were plated and incubated at 28 °C to determine the total number of CFUs and at 37 °C to determine the number of CFUs carrying integrated plasmid.

To test the stability of integration, HKU09-01:pPI03 was grown for 4 consecutive subcultures (diluting 1 in 1000 each time) at 37 °C or 28 °C without antibiotic selection. Dilutions were plated on TSA and 50 colonies were screened for chloramphenicol resistance.

2.4 Isolation of *S. lugdunensis* HKU09-01 $\Delta recA$

The *recA* mutation in HKU09-01 was isolated by allelic replacement using the thermosensitive plasmid pIMAY (Monk et al., 2012). 500 bp fragments upstream and downstream of the *recA* gene were PCR-amplified and assembled using spliced overlap extension PCR. The resulting 1 kb deletion cassette was cloned into pIMAY and used to replace the chromosomal *recA* gene by allelic exchange (Heilbronner et al., 2013; Monk et al., 2012).

2.5 Growth curves

Growth kinetics of different strains was assessed using the SynergyH1 Hybrid reader (BioTek) in 96 well plates (Nunc). Triplicate wells were filled with 200 µl TSB inoculated to an OD₅₇₈ = 0.05 with a fresh overnight culture. The optical density at 578 nm was measured every 45 min with

10 min of shaking prior to every read. Each growth curve was recorded three times using independent overnight starter cultures.

2.6 UV sensitivity

Colonies were picked from a fresh plate and streaked on a new TSA plate. Plates were exposed for indicated times to short wave UV light using the “Mineralight Lamp” Model CC10 (UVP) in a Chomato-VUE Cabinet. Plates were incubated for 24 h at 37 °C and growth was evaluated semi quantitatively.

2.7 Competitive growth experiments

Two strains (labelled with pIPIkan or pIPIery) were grown in TSB_{cm10} overnight. Cells were harvested (10 min at 9000 x g), washed with TSB and adjusted to an OD₅₇₈ = 3. 50 µl of each strain was inoculated into 10 ml TSB_{cm10}. Dilutions were plated on TSA_{IPTG/Kan} (1 mM IPTG + 50 µg / ml kanamycin) and TSA_{IPTG/Ery} (1 mM IPTG + 10 µg / ml erythromycin) to determine the ratio of cells at the start of the experiment (C0). The culture was incubated for 24 h with shaking at 200 rpm at 37 °C. The OD₅₇₈ of the overnight culture was measured and the approximate number of generations was calculated. Dilutions were plated on TSA_{IPTG/Kan} and TSA_{IPTG/Ery} to determine the ratio of the strains after 24 h growth (C1). A new TSB culture was inoculated to an OD₅₇₈ = 0.01 with the previous culture and incubated at 37 °C for another 24 h. The process was repeated four times and the ratio of strains in the consecutive cultures was determined.

3. Results

3.1 The phage-integrase vector pIPI03

S. lugdunensis N920143 carries the lysogenic phage ϕ SL1 (Heilbronner et al., 2011). The phage is integrated into gene SLUG_08250 which encodes a putative phosphosugar transport protein. Strain HKU09-01 does not harbour the phage (Heilbronner et al., 2011; Tse et al., 2010).

To create a vector for the site-specific chromosomal integration, the integrase gene *ccrB* and the corresponding attachment site *attP* were amplified and cloned into the thermosensitive vector pIMAY (Monk et al., 2012) to form pPII03 (Fig. 1A). The *cat* and *ccrB* genes form a transcriptional fusion and are constitutively expressed.

To be able to distinguish between strains with the same genetic background, two different variants of pPII03 were constructed by cloning IPTG-inducible antibiotic resistance determinants (Monk et al., 2008a) into the multiple cloning site of the plasmid. pPII_{ery} confers inducible erythromycin resistance and pPII_{kan} confers kanamycin resistance (Fig. 1B). The inducible cassettes contain a Phelp promoter followed by a LacO repressor binding site and the resistance determinant. The *lacI* (repressor) coding sequence is present and its expression is driven by a second Phelp promoter. Thus the antibiotic resistances are repressed unless induced by IPTG.

3.2 Integration of pPII03

S. lugdunensis HKU09-01 was transformed with pPII03 with selection on Cm agar at 28 °C, the permissive temperature for plasmid replication. Subsequent plating at 37 °C allowed the selection of clones with an integrated plasmid. Determination of the total number of CFUs grown at 28 °C and 37 °C revealed that 0.09 % to 0.13 % of cells present within a colony carried the plasmid integrated into the chromosome.

PCR experiments using primer combinations recognizing sequences in the chromosome and the pPII03 backbone (Fig. 2) confirmed that the plasmid had integrated at the same site as phage ϕ SL01 in N920143. The PCR1 experiment using the chromosomal primers scr.F and scr.R yielded a 600 bp fragment with DNA isolated from HKU09-01 (pPII03) grown on 28 °C, showing that the phage attachment site is not occupied in this strain (Fig. 2B). No product was formed with DNA from

HKU09-01::pIP103 grown on 37 °C, indicating that the site is occupied by the integrated plasmid. The PCR2 experiment used primers ccrB. F and attP. R and confirmed the presence of replicating plasmid in HKU09-01 (pIP103). PCR2 failed to detect autonomous plasmid in HKU09-01::pIP103 (Fig. 2B). The primers used in PCR 3 (scr.R and attP.R) bound to sites in the chromosome and the plasmid, respectively, and yielded a 400 bp fragment with DNA from HKU09-01::pIP103. This confirmed the site-specific integration of the plasmid. Interestingly, this PCR produced a faint product with HKU09-01 (pIP103) as well (Fig. 2B), showing that at growth on 28 C° a small subpopulation of cells carried the plasmid integrated in the chromosome.

The stability of the integrated form of pIP103 was investigated. No loss of Cm resistance (<2%) was detected when strains with integrated plasmid were grown for four consecutive subcultures (ca. 40 generations) in TSB without antibiotic pressure at 28 °C and at 37 °C. To make sure that the integrated plasmid was maintained, 10 representative colonies (after growth on 28 °C and 37 °C) were screened by PCR using the primers described above. All clones showed integrated plasmid and the PCR tests failed to detect autonomous plasmid (data not shown). These results suggest that the phage integrase of ϕ SL01 is only responsible for the insertion of the plasmid into the chromosome and not for its excision.

Recently MLST typing was established for *S. lugdunensis* (Chassaïn et al., 2012) and revealed five clonal complexes (CCs). PCR1 experiments (using primers scr.F and scr.R) showed that none of the 10 strains in our collection representing isolates of all CCs carried a lysogenic phage at the chromosomal *attB* site of ϕ SL1 (data not shown). This prompted us to investigate whether pIP103 is able to integrate into the *attB* site of strains from different CCs. Since HKU09-01 represents a member of CC1 we chose five isolates representing other CCs (SL71 (CC2), SL27 (CC3), SL37 (CC3), SL62 (CC4) and SL81(CC5)). All strains could be transformed with pIP103 and the plasmid integrated specifically into the *attB* site as confirmed by PCR3 using the primer combination scr.R and attP.R (data not shown).

3.3 Effects of pPII03 integration on bacterial fitness

The *ccrB* gene in pPII03 is expressed constitutively by the *Phelp* promoter. We performed growth curve analysis to exclude the possibility that this influences bacterial fitness. At 37 °C the HKU09-01 wild-type and HKU09-01::pPII03 strains showed identical lag phases (ca. 2.5h) and similar exponential growth rates (generation times of 98 min and 93 min, respectively) (Fig. 3A). This shows that *ccrB* expression did not have any toxic effects. However, when the strains were grown at a temperature that allowed integrated plasmid to begin replication (28 °C), the strain had a significantly slower rate of growth (Fig. 3B). At 28 °C both strains showed a lag phase of ca. 4 h 20 min. However, the generation time of the wild-type strain was 181 min, while it was 246 min for the strain with the integrated plasmid. This is most likely due to the thermosensitive replication machinery of pPII03 attempting to replicate the integrated plasmid which subsequently interferes with the chromosomal replication.

3.4 Integration of pPII03 is *recA* independent

A *recA* mutant of HKU09-01 was tested to determine if integration of pPII03 is independent of RecA-promoted recombination. RecA is known to be an important mediator of the bacterial SOS response and *recA* mutants have a reduced ability to repair damaged DNA using recombination by RecA-dependent recombination. As expected, the $\Delta recA$ mutant displayed strongly increased sensitivity to UV-light. The wild-type strain could tolerate UV-light exposure for up to 20 seconds without visible effects on growth, while the $\Delta recA$ mutant showed reduced viability even after only 5 seconds of exposure (Fig. 4A). In a growth curve experiment the $\Delta recA$ mutant displayed a longer lag phase (3 h 10 min) and a reduced growth rate (163 min per generation) compared to the wild-type strain (Fig. 4B).

The integration of pPII03 occurred independently of the *recA* genotype because pPII03 integrants occurred at the same frequency in the *recA* mutant as in the wild-type (data not shown).

This supports the idea that CcrB does not need RecA-mediated processes to mediate integration into the chromosome.

3.5 pPI03 in competitive growth experiments

Plasmids pPI03_{ery} and pPI03_{kan} were integrated into the chromosome of different strains to facilitate competitive growth experiments and to discriminate between strains growing in the same culture. Both plasmids were integrated into the HKU09-01 wild-type strain, creating HK(WT):Ery and HK(WT):Kan. TSB was inoculated with the two strains at a ratio of 1:1, grown to saturation, diluted in fresh broth, grown again to saturation. The process was continued for four consecutive cultures. The ratio of erythromycin to kanamycin resistant cells was determined at the beginning and after each of the subcultures. The results are shown in Fig. 5 (red line). The ratio of erythromycin : kanamycin resistant bacteria remained constant over the course of the experiment. The only difference between the strains was the nature of the resistance determinant and it can be concluded that this did not influence the growth of the bacteria in the competition experiment.

The $\Delta recA$ mutant showed a growth defect in the growth curve analysis (Fig. 4B). We integrated both plasmids into the *recA*⁻ strain, creating HK(R):Ery and HK(R):Kan and compared the strains in competitive growth experiments. No differences were observed in the outcome when HK(R):Ery vs. HK(WT):Kan was compared to HK(R):Kan vs. HK(WT):Ery. Therefore the experiments were combined to one data set HK(R) vs HK(WT) (blue line in Fig. 5).

In contrast the data demonstrated a strong growth advantage for the wild-type compared to the *recA* mutant. The two strains started the experiment at a ratio of 1:1. However the *recA* mutant was rapidly outcompeted and was present at a 10⁵ fold lower number at the end of the experiment.

4. Discussion

Site-specific integrative plasmids are important tools for modern molecular microbiology since they allow the efficient insertion of DNA in a specific genomic region. Phage-integrase vectors represent a universal class of such vectors and can be constructed for every species. The *ccrB* gene together with the corresponding *attP* site of any phage can be used for the construction of integrative plasmids for any species as long as the strain to be tested is not lysogenic and the CcrB protein does not promote excision of the phage. The phage-integrase vector will integrate stably into the bacterial chromosome.

We have constructed a site-specific integration vector for *S. lugdunensis* using the coding sequence of the CcrB integrase and the corresponding *attP* site of phage ϕ SI1 (Heilbronner et al., 2011). The plasmid was found to integrate specifically, irreversibly and *recA*-independently into the *attB* site of HKU09-01. The constitutive expression of *ccrB* in this vector did not have a negative effect on bacterial fitness. However, the integrated plasmid led to a reduction in growth rate at 28 °C. This is most likely due to the thermosensitive nature of the plasmid replication system, which allows replication to be attempted at 28 °C which could interfere with the chromosomal replication. This should be borne in mind when pIPI03 is employed to complement mutants, for example. Despite this no plasmid loss was detected after 40 generations of growth at 28°C. Apart from N920143, none of the *S. lugdunensis* strains from CC1-CC5 harbored a lysogenic phage in the *attB* site. Consequently we were able to integrate pIPI03 into the chromosome of each strain. This demonstrates the broad range of *S. lugdunensis* strains that can conveniently be labeled using pIPI03.

There are many possible applications of site-specific integrative plasmids such as pIPI03. The extensive multiple cloning site of pIPI03 facilitates insertion of DNA fragments into the plasmid. We cloned two IPTG-inducible antibiotic resistance cassettes into pIPI03 and demonstrated their use for competitive growth experiments. Competitive growth is a powerful technique to investigate the effects of the controlled expression of a single gene on the cell physiology under identical growth conditions and can be used both *in vitro* and *in vivo* (Monk et al., 2008a). pIPI_{ery} and pIPI_{kan} allowed

easy labeling of *S. lugdunensis* strains with clearly distinguishable phenotypes and hence the accurate determination of the ratio of the two strains in competitive growth experiments. It is possible to detect subtle differences by prolonged competitive growth when differences in growth rate/yield in a conventional growth curve experiment would not be seen. Furthermore, the growth of different strains in the same culture allows the accurate analysis of environmental stress factors, such as antibacterial compounds or nutrient limitation and allows investigation of their effects on strains under direct competition.

Another application of integration vectors is complementation of mutants. This would be particularly attractive for experiments involving growth in experimental animals where selection for an autonomously replicating plasmid is difficult and where plasmid loss is magnified by stressful conditions of growth *in vivo*. Furthermore complementation may be distorted by the gene-dosage effect of expressing the wild-type gene from a multicopy plasmid. The integrated vector is very stable and provides expression from a single copy of the wild-type gene.

The *S. lugdunensis* integrative plasmid pIP03 is ideally suited for these applications and will facilitate future experiments to allow a deeper understanding of the virulence potential of this neglected coagulase negative organism

5. Nucleotide sequence accession number

The nucleotide sequence of pIP03 was deposited in GenBank under accession number XXXXX

6. Acknowledgements

We acknowledge the support of the Irish Research Council for Science, Engineering and Technology for an Embark scholarship (RS2000192) and Science Foundation Ireland for a Programme Investigator grant (08/IN.1/B1854) (to TJF). We thank Martine Pestel-Caron for supplying *S. lugdunensis* strains from different clonal complexes.

7. References

- Anguera, I., et al., 2005. *Staphylococcus lugdunensis* infective endocarditis: description of 10 cases and analysis of native valve, prosthetic valve, and pacemaker lead endocarditis clinical profiles. *Heart*. 91, e10.
- Bieber, L., Kahlmeter, G., 2010. *Staphylococcus lugdunensis* in several niches of the normal skin flora. *Clin Microbiol Infect*. 16, 385-8.
- Chassaïn, B., et al., 2012. Multilocus sequence typing analysis of *Staphylococcus lugdunensis* implies a clonal population structure. *J Clin Microbiol*. 50, 3003-9.
- Freney, J., et al., 1988. *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., Two Species from Human Clinical Specimens. *Int J Syst Bacteriol*. 38, 168-172.
- Heilbronner, S., et al., 2013. Sortase A promotes virulence in experimental *Staphylococcus lugdunensis* endocarditis. *Micobiology*. (Submitted).
- Heilbronner, S., et al., 2011. Genome sequence of *Staphylococcus lugdunensis* N920143 allows identification of putative colonization and virulence factors. *FEMS Microbiol Lett*. 322, 60-7.
- Hirano, N., et al., 2011. Site-specific recombinases as tools for heterologous gene integration. *Appl Microbiol Biotechnol*. 92, 227-39.
- Lei, M. G., et al., 2012. A single copy integration vector that integrates at an engineered site on the *Staphylococcus aureus* chromosome. *BMC Res Notes*. 5, 5.
- Li, M. Z., Elledge, S. J., 2007. Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC. *Nat Methods*. 4, 251-6.
- Li, M. Z., Elledge, S. J., 2012. SLIC: a method for sequence- and ligation-independent cloning. *Methods Mol Biol*. 852, 51-9.
- Monk, I. R., et al., 2008a. Development of multiple strain competitive index assays for *Listeria monocytogenes* using pIMC; a new site-specific integrative vector. *BMC Microbiol*. 8, 96.
- Monk, I. R., et al., 2008b. Tools for functional postgenomic analysis of *Listeria monocytogenes*. *Appl Environ Microbiol*. 74, 3921-34.

324 Monk, I. R., et al., 2012. Transforming the untransformable: application of direct transformation to
325 manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. MBio. 3.
326 Patil, R., et al., 2011. *Staphylococcus lugdunensis* native tricuspid valve endocarditis: a case report
327 and review of literature. J Gen Intern Med. 26, 1209-11.
328 Petersen, K. V., et al., 2013. Repetitive marker-free site-specific integration - a novel tool for multiple
329 chromosomal integration of DNA. Appl Environ Microbiol.
330 Tse, H., et al., 2010. Complete genome sequence of *Staphylococcus lugdunensis* strain HKU09-01. J
331 Bacteriol. 192, 1471-2.
332 Vandenesch, F., et al., 1993. Endocarditis due to *Staphylococcus lugdunensis*: report of 11 cases and
333 review. Clin Infect Dis. 17, 871-6.
334 Zapotoczna, M., et al., 2012. Iron-Regulated Surface Determinant (Isd) Proteins of *Staphylococcus*
335 *lugdunensis*. J Bacteriol. 194, 6453-67.
336
337
338

Figures and tables:

Fig. 1.

(A) Schematic diagram of pIPI03

The coding sequences of the thermosensitive plasmid pIMAY are shown in white. Coding sequences of the ϕ SL1 integrase (*ccrB*) and the corresponding attachment site (*attP*) are shown in black. Unique restriction sites are indicated. For sequencing purposes, T3 and T7 primer binding sites are present before the *KpnI* and after the *SacI* restriction sites, respectively.

(B) Schematic diagram of the IPTG-inducible antibiotic resistance cassettes.

These were cloned into the MCS of pIPI03 using *SacI* and *KpnI*. *ermAM* – erythromycin resistance; *aphA3* – kanamycin resistance.

Fig. 2.

(A) Schematic diagram of the integration of pIPI03.

The plasmid-encoded genes are shown in white and the *attP* site on the plasmid is shown in blue. The gene SLUG08250 harboring the *attB* site (red) in the HKU09-01 chromosome is shown in tan. Arrows indicate the location of primers for the detection of integration by PCR. Integration of pIPI03 disrupts the attachment sites resulting in $\frac{1}{2}$ *attB* and $\frac{1}{2}$ *attP* on either side of the integrated plasmid.

(B) PCR to detect the integration of pIPI03.

Primer combination 1 (scr. F / scr. R) was used to determine if the chromosomal *attB* site was occupied or not. Primer combination 2 (*ccrB*. F / *attP*. R) was used to detect autonomous plasmid. Primer combination 3 (*attP*. R / scr. R) determined if pIPI03 was integrated into the *attB* site in the chromosome.

Fig. 3.

(A) Growth curves of HKU09-01 and HKU09-01::pPI03 (integrated plasmid) on 37 °C. The mean and standard deviation of three independent growth curves is shown.

(B) Growth curves of HKU09-01 and HKU09-01::pPI03 (integrated plasmid) at 28 °C. The mean and standard deviation of three independent growth curves is shown.

Fig 4.

(A) Semi quantitative determination of UV sensitivity displayed by HKU09-01 and the isogenic $\Delta recA$ mutant. Exposure to short wave UV light is given in seconds. Growth was assessed after 24 hours of incubation. +++ – normal growth; ++ – weakly impaired growth; + – strongly impaired growth; - – no growth

(B) Growth curves of HKU09-01 and the isogenic $\Delta recA$ mutant at 37 °C. The mean and standard deviation of three independent growth curves is shown.

Fig. 5.

Competitive growth experiment

TSA cultures were inoculated with two strains (labeled with pPI_{kan} and pPI_{ery}, respectively) and grown through four consecutive subcultures. CFUs were determined at stationary phase at each stage and the ratio of erythromycin : kanamycin resistant cells was calculated. The mean and standard deviation of three independent experiments is shown. The red line represents competitive growth of HKU09-01(WT):Ery and HK(WT):Kan. The number of erythromycin resistant CFUs was set to 1 and the number of kanamycin resistant CFUs was expressed in relation to this value. The blue line represents competitive growth of HK(WT) and the isogenic $\Delta recA$ mutant HK(R). The number of HK(R) CFUs was set to 1 and the number of HK(WT) CFUs was expressed in relation to this value.

Bacterial strain or plasmid	Description	Reference / Source
<i>Staphylococci</i>		
<i>S. lugdunensis</i> N920143	Human clinical isolate, genome sequenced, CC1	(Heilbronner et al., 2011)
<i>S. lugdunensis</i> HKU09-01	Clinical isolate, genome sequenced to completion, CC1	(Chassaïn et al., 2012; Tse et al., 2010)
<i>S. lugdunensis</i> SL2	Clinical isolate CC2	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL9	Clinical isolate CC5	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL13	Clinical isolate CC1	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL27	Clinical isolate CC4	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL37	Clinical isolate CC3	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL57	Clinical isolate CC3	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL62	Clinical isolate CC4	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL71	Clinical isolate CC2	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL72	Clinical isolate CC1	(Chassaïn et al., 2012)
<i>S. lugdunensis</i> SL81	Clinical isolate CC5	(Chassaïn et al., 2012)
<i>E.coli</i>		
DH10B	<i>dam</i> ⁺ <i>dcm</i> ⁺ Δ <i>hsdRMS</i> <i>endA1</i> <i>recA1</i> high efficiency cloning strain	Invitrogen
Plasmids		
pIMAY	Thermosensitive vector for allelic exchange	(Monk et al., 2012)
pIMAY- Δ <i>recA</i>	A deletion encompassing a 700 bp internal fragment of <i>recA</i> amplified from HKU09-01	This study
pIPI03	Site-specific integration vector for HKU09-01	This study
pIPI03 <i>ery</i>	pIPI03 carrying IPTG-inducible <i>ermAM</i> genes from pIMC <i>ery</i> within the MCS	This study
pIPI03 <i>kan</i>	pIPI03 carrying the IPTG-inducible <i>aphA3</i> gene from pIMC <i>kan</i> within the MCS	This study
pIMC <i>ery</i>	Site-specific integration vector for <i>L. monocytogenes</i>	(Monk et al., 2008a)
pIMC <i>kan</i>	Site-specific integration vector for <i>L. monocytogenes</i>	(Monk et al., 2008a)

389 Table 2 Oligonucleotides used in this study

Primer	5'-3' Sequence	Restriction site / SLIC homology
ΔrecA-A	at ~~tttGagCt~~ Ctaacatgaaattaataagtttacc	SacI
ΔrecA-B	Cctaaagcattatctaagtgcactgatcc	
ΔrecA-C	cattagataatgctttaggctaaaagaaaatccagaagtaagag	
ΔrecA-D	ACAAACgAATTcTACTTTTTAGTTGTGT	EcoRI
ΔrecA-Sc. F	ggttctgatgatattcttatagaagaagc	
ΔrecA-Sc. R	atctaaatttcctcttttgaagaagtc	
ccrB F	<u>GATAGGCCTAATGACTGGCTTTTATAA</u> AGGAGgTGAAACA	pIMAY overlap (behind cat)
	ATGAAAAGTAG	
attP R	<u>AAAAAGTACAGTCGGCATTATCTCATAGCATGCATATAATT</u>	pIMAY overlap (behind cat)
	TTTTAAATG	
pIMAY_SLICcat_ F	TTATAAAAGCCAGTCATTAGGCCTATCTGAC	
pIMAY_SLICcat_ R	TATGAGATAATGCCGACTGTACTTTTACAG	
attB Sc.-F	CCCTTTATCTGCAGTTATCTTAATTTATATGGC	
attB Sc.-R	GACCAAAAAGCAAAACCGGTACCC	

390

391

392

393

394

Fig. 1.tif
[Click here to download high resolution image](#)

A

B

Fig. 2.tif
[Click here to download high resolution image](#)

A

B

Fig. 3.tif
[Click here to download high resolution image](#)

Fig. 4.tif
[Click here to download high resolution image](#)

A

	WT	<i>recA</i> ⁻
0 s	+++	+++
5 s	+++	++
10 s	+++	+
20 s	+++	-
30 s	++	-
60 s	-	-

B

Fig. 5.tif
[Click here to download high resolution image](#)