Development of a genetic system for a model manganese-oxidizing proteobacterium, *Leptothrix discophora* SS1

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Understanding the molecular underpinnings of manganese oxidation in *Leptothrix discophora* SS1 has been hampered by the lack of a genetic system. In this report, we describe the development of a genetic system for *L. discophora* SS1. The antibiotic sensitivity was characterized, and a procedure for transformation with exogenous DNA via conjugation was developed and optimized, resulting in a maximum transfer frequency of 5.2 × 10⁻⁴ and a typical transfer frequency of the order of 1 × 10⁻⁵ transconjugants per donor. Genetic manipulation of *L. discophora* SS1 was demonstrated by disrupting *pyrF* via chromosomal integration with a plasmid containing a R6K⁺ conditional origin of replication through homologous recombination. This resulted in resistance to 5-fluoroorotidine, which was abolished by complementation with an ectopically expressed copy of *pyrF* cloned into pBBR1MCS. This system is expected to be amenable to a systematic genetic analysis of *L. discophora* SS1, including those genes responsible for manganese oxidation.

**INTRODUCTION**

*Leptothrix discophora* SS1 is a filamentous Gram-negative betaproteobacterium best known for its ability to oxidize soluble manganese (II) to insoluble manganese oxides (III and IV) (Ghiorse, 1984; Nea1son et al., 1988; Tebo et al., 1997). Although several enzymes responsible for manganese oxidation in other bacteria have been reported (Francis & Tebo, 2002; Ridge et al., 2007; Anderson et al., 2009; Butterfield et al., 2013; Su et al., 2013), the molecular determinants of manganese oxidation by *L. discophora* SS1 are poorly characterized. Our understanding of manganese oxidation by this strain has been hampered by the lack of a genetic system. Studies of *L. discophora* SS1 morphology, physiology and biochemistry that did not require a genetic system have increased our knowledge about the biology of this micro-organism (Adams & Ghiorse, 1986, 1987) and its so-called manganese oxidizing factor (MOF). However, attempts to clone and heterologously express the genes thought to encode MOF did not result in a protein that oxidized manganese (Brouwers, 1999; El Gheriany, 2010).

Despite previous efforts to develop a genetic system for *L. discophora* SS1 (Siering, 1996), no successful introduction of exogenous DNA has been reported. To move forward in the study of manganese oxidation by this organism, it seems imperative that a genetic system be developed.

Here, for the first time, to our knowledge, we report on the development of a genetic system for *L. discophora* SS1. We showed that the transfer of exogenous DNA into this micro-organism via conjugation is possible at rates comparable to those of other Gram-negative bacteria (Dahliberg et al., 1998; Coppi et al., 2001; Hao et al., 2012), although the rates were highly variable from experiment to experiment. We demonstrated that a suicide plasmid (pVIK165), containing the widely used R6K⁺ conditional origin of replication, was able to integrate into the *L. discophora* SS1 chromosome via homologous recombination, thus allowing us to insert a copy of *pyrF*, whose product is required for uracil auxotrophy and 5-fluorouracil sensitivity (Isaac & Holloway, 1968; Thia-Toong et al., 2002). Finally, we complemented the *pyrF* insertion mutant in trans with a plasmid-borne copy of *pyrF* that restored uracil auxotrophy and 5-fluorouracil sensitivity. This system should allow for a more systematic analysis of the genetic underpinnings of *L. discophora* SS1 biology, including manganese oxidation.

**Abbreviations**: 5-FOA, 5-fluoroorotidine; MOF, manganese oxidizing factor; OMP, orotidine monophosphate; UMP, uridine monophosphate.

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Methods

Culture conditions and growth media. *L. discophora* SS1 was maintained on 2× PYG medium (Adams & Ghiorse, 1986) (0.5 g peptone l⁻¹, 0.5 g yeast l⁻¹, 0.5 g glucose l⁻¹, 0.6 g MgSO₄, 7H₂O l⁻¹, 0.07 g CaCl₂ l⁻¹ and 3.6 g HEPES l⁻¹, adjusted to pH 7.2 with 1 M NaOH) either on plates or poured tubes. Transfers to fresh plates were made every 2 weeks. Cultures for most experiments were grown in 60 ml glass tubes containing 20 ml 2× PYG in 250 ml glass flasks. Minimum salts vitamin glucose medium [MSVG: 0.24 g (NH₄)₂SO₄ l⁻¹, 0.06 g MgSO₄, 7H₂O l⁻¹, 0.07 g CaCl₂ l⁻¹, 0.02 g KH₂PO₄ l⁻¹, 0.03 Na₂HPO₄ l⁻¹, 2.4 g HEPES l⁻¹ and 0.5 g glucose l⁻¹, adjusted to pH 7.2 with 1 M NaOH] (Adams & Ghiorse, 1986) was also used and amended with 1% Casamino acids and/or 50 μg uracil ml⁻¹ when needed. Antibiotics were used as needed at the following concentrations: 50 μg kanamycin ml⁻¹, 15 μg nalidixic acid ml⁻¹, 50 μg rifampicin ml⁻¹, 50 μg streptomycin ml⁻¹, 10 μg tetracycline ml⁻¹, 10 μg gentamicin ml⁻¹, 12.5 μg chloramphenicol ml⁻¹ and 100 μg ampicillin ml⁻¹. 5-Fluorooroticid (5-FOA) was prepared as a 1000× DMSO stock solution and used at a concentration of 50 μg ml⁻¹ when needed.

Assessment of *L. discophora* SS1 sensitivity to antibiotics and isolation of spontaneous antibiotic-resistant mutants. We analysed the susceptibility of *L. discophora* SS1 to antibiotics by spreading 100 μl stationary-phase culture of *L. discophora* SS1 on 2× PYG plates, on top of which we applied sterile filter paper discs soaked in antibiotic (Table 1). Next, we determined MICs in liquid cultures by testing growth under several dilutions of each antibiotic. MICs in liquid cultures were: rifampicin, 5 μg ml⁻¹; ampicillin, 1 μg ml⁻¹; kanamycin, 1 μg ml⁻¹; tetracycline, 0.5 μg ml⁻¹; gentamicin, 1 μg ml⁻¹; chloramphenicol, 0.5 μg ml⁻¹; streptomycin/spectinomycin, 1 μg ml⁻¹; and nalidixic acid, 0.5 μg ml⁻¹.

To isolate nalidixic acid-resistant mutants, a 10 ml aliquot of an actively growing culture of *L. discophora* SS1 was inoculated into 40 ml 2× PYG. This culture was allowed to reach exponential phase (~2 days) at which point nalidixic acid was added. This culture was shaken at 120 r.p.m. for ~7 days, after which it was spun down, the supernatant was removed and the cells were inoculated into 50 ml fresh 2× PYG-nalidixic acid. This culture was allowed to grow for 7 days at which point 5 ml was removed, concentrated to 100 μl and spread onto 2× PYG-nalidixic acid plates. Colonies that appeared after ~1 week were restreaked onto fresh antibiotic-containing plates and upon confirmation of their resistance, a single colony was chosen for further experiments. Subsequently, a similar approach was used to isolate rifampicin and nalidixic acid double mutants.

Optimization of conjugation

Age of the recipient. To assess the effect of recipient’s age on conjugation efficiency, 10 replicates of *L. discophora* SS1 were grown (20 ml each) and two were used for each time point: early exponential phase (12–18 h), mid-exponential phase (18–34 h), late exponential phase (34–40 h), stationary phase (40–48 h) and late stationary phase (48–60 h). The donor culture, *Escherichia coli* S17 pBRR1MCS2, was refreshed at appropriate intervals such that each mating mix contained donor of the same optical density and age.

Donor/recipient ratio. To assess the effect of donor/recipient ratio on conjugation, the donor (*E. coli* S17) and recipient (*L. discophora* SS1) were grown to mid-exponential phase (OD₆₀₀ 0.4 for *E. coli*) and late exponential phase (OD₆₀₀ 0.25 for *L. discophora* SS1), respectively. The OD₆₀₀ of a 200 μl aliquot was measured in a 96-well plate using a Synergy plate reader (BioTeck). Counts (c.f.u.) were determined by plating serial dilutions of these cultures. Next, 10 ml *E. coli* and 100 ml *L. discophora* SS1 were concentrated 10 and 100 times, respectively, by centrifugation (Beckman TJ-6) at 2700 r.p.m. for 15 min at room temperature. These thick suspensions were used to obtain the ratios indicated in Table 4 in a final volume of 500 μl.

Mating time. Six identical mating mixtures were prepared as described, and were allowed to mate (in duplicate) for 6, 12 and 18 h, after which they were plated as described above.

Recovery after mating. Two identical mating mixtures were set up and mated for the same amount of time and under the same conditions, after which one of the mating mixtures was resuspended in 1 ml 2× PYG and dilutions plated immediately. The other replicate was resuspended in 20 ml liquid 2× PYG without antibiotics and allowed to recover for 6–8 h by shaking on a rotary shaker at 120 r.p.m. Cells were then collected by centrifugation, resuspended in 1 ml 2× PYG and dilutions were plated.

Table 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Species or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. discophora</em> SS1</td>
<td>WT</td>
<td>Adams &amp; Ghiorse (1986)</td>
</tr>
<tr>
<td><em>L. discophora</em> SS1 Nal⁺</td>
<td>Spontaneous rifampicin/nalidixic acid-resistant mutant</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. discophora</em> SS1 Nal⁺ pJSpyrF</td>
<td>pyrF interrupted mutant</td>
<td>This work</td>
</tr>
<tr>
<td><em>L. discophora</em> SS1 Nal⁺ pJSpyrF pBBpyrF</td>
<td>pyrF interrupted mutant complemented with the WT copy of pyrF</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1 zip (E. coli S17)</td>
<td>zip lysogen</td>
<td>Koller &amp; Helsinki (1979)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBRR1MCS</td>
<td>Broad-host range vector, Cm resistant</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pBRR1MCS-2</td>
<td>Broad-host range vector, Km resistant</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pVK165</td>
<td>Suicide plasmid, Km resistant</td>
<td>Kalogeraki &amp; Winans (1997)</td>
</tr>
<tr>
<td>pJSpyrF</td>
<td>pVK165 containing pyrF fragment (used for homologous recombination)</td>
<td>This work</td>
</tr>
<tr>
<td>pBBpyrF</td>
<td>pBRR1MCS containing purBpyrF (complementation plasmid)</td>
<td>This work</td>
</tr>
</tbody>
</table>

Cm, chloramphenicol; Km, kanamycin.
**Table 2. Primers used**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrF int For SacI</td>
<td>ATATATGAGCTCACGCCAGCAAGATCTTCGACTCTT</td>
<td>Amplifies an internal fragment of pyrF (~600 bp)</td>
</tr>
<tr>
<td>pyrF int Rev XbaI</td>
<td>ATATATTCCTAGACGCGGAGCTTGTTGACGATGA</td>
<td>Amplifies the entire length of pyrF together with purB including the native promoter (~3 kb)</td>
</tr>
<tr>
<td>pyrFpurB For KpnI</td>
<td>ATATGGACCTGTTGAGAAAGACTGTCGTTGA</td>
<td>Located on plasmid, used to confirm plasmid integration</td>
</tr>
<tr>
<td>pyrFpurB Rev SacI</td>
<td>ATATATGAGCTCAGTAACGTGCAGCGACCA</td>
<td>Amplifies the entire pyrF gene (~800 bp)</td>
</tr>
<tr>
<td>GFP Rev</td>
<td>ATTTGTATAGTTCATCCATGC</td>
<td>Located on plasmid, used to confirm plasmid integration</td>
</tr>
<tr>
<td>pyrF For</td>
<td>ATGAACCTACCGGACCCAG</td>
<td>Located on plasmid, used to confirm plasmid integration</td>
</tr>
<tr>
<td>pyrF Rev</td>
<td>CTGGTGCCTGTCGACTT</td>
<td>Located on plasmid, used to confirm plasmid integration</td>
</tr>
</tbody>
</table>

**Effect of manganese on conjugation.** Mating mixtures were always plated in parallel on plates containing manganese and plates without manganese for all of the conditions assessed. Transconjugants were always replated onto manganese to verify that they were *L. discophora*, although this confirmation could have been done by other means, such as PCR of *L. discophora*-specific genes.

**Plasmid stability in *L. discophora* SS1.** *L. discophora* SS1 NaI+ pBBR1MCS2 was initially scraped from plates, inoculated into 20 ml 2 × PYG with appropriate antibiotics and allowed to grow to late exponential phase, and then 5 ml culture was transferred to 15 ml fresh medium containing antibiotics. When this culture reached late exponential phase, it was diluted 10-fold with 2 × PYG medium with or without kanamycin and allowed to grow for 2 days, after which time serial dilutions were spread onto plates with and without kanamycin. Similar transfers from the culture grown without antibiotics to media with and without antibiotics were performed every 2 days for six transfers spanning 18 days. This experiment was performed in triplicate. Duplicate dilutions of each replicate were spread onto plates and c.f.u. were counted to assess plasmid stability with and without antibiotic selection.

**Plasmid construction/DNA manipulations.** SacI and XbaI restriction sites were added to the forward and reverse primers, respectively, to facilitate downstream cloning. Primers pyrF int For SacI and pyrF int Rev XbaI (Table 2) were used to amplify a 640 bp internal fragment of pyrF from *L. discophora* SS1.

The resulting fragment was digested with SacI and XbaI, and then cloned into the SacI/XbaI sites of pVJK165 to create plasmid pJSpyrF. This plasmid was transformed into *E. coli* S17, and one colony that harbouring the plasmid was further used as a donor in a mating experiment. To create the complementation plasmid, a 3 kb fragment of DNA including both pyrF and purB (to include their native promoter located upstream of purB) was amplified from *L. discophora* SS1 using primers pyrFpurB For KpnI and pyrFpurB Rev SacI. The PCR product was cleaned, digested and ligated into the KpnI and SacI sites of pBBR1MCS, generating plasmid pBBpyrF. This plasmid was transformed into *E. coli* S17- and one chloramphenicol-resistant clone harbouring the plasmid was further used as a donor in a mating experiment with *L. discophora* SS1 NaI+. pJSpyrF to create *L. discophora* SS1 NaI+; pJSpyrF pBBpyrF (hereafter referred to as the complement strain).

**Assessment of uracil auxotrophy/prototrophy.** *L. discophora* SS1 NaI+; pJSpyrF and the complement strain were first grown to late exponential phase on 2 × PYG-kanamycin/5-FOA. A 500 µl aliquot was centrifuged to remove the supernatant. Cells were washed with 500 µl MSVG prior to inoculation into fresh MSVG with or without 50 µg uracil ml⁻¹ and growth was monitored via optical density.

**RESULTS AND DISCUSSION**

**Isolation of spontaneous antibiotic-resistant mutants**

Antibiotic markers are the building blocks of a genetic toolbox (Bitan-Banin et al., 2003). *L. discophora* SS1 showed zones of inhibition for all 11 antibiotics tested (Table 3). The results from the MIC tests indicated that...
L. discophora SS1 was sensitive to nalidixic acid, rifampicin and streptomycin, amongst other antibiotics. We readily isolated mutants that were resistant to each of these and that could be used during counter-selection against E. coli donors during conjugation. The growth pattern of an isolated spontaneous nalidixic acid-resistant mutant was similar to that of the WT L. discophora SS1 for the working concentration tested (Fig. 2), and we subsequently used this antibiotic-resistant mutant to develop a genetic system for this micro-organism.

The MIC results also indicated that L. discophora SS1 was sensitive to several widely used antibiotics such as chloramphenicol, kanamycin and tetracycline for which plasmid-borne resistance markers are available that could be used in downstream genetic manipulations.

### Efficient DNA transfer into L. discophora SS1 by conjugation

Previous attempts to establish a DNA transfer protocol via electroporation or chemical competence by Siering (1996) were not successful, most likely due to the low survival of L. discophora SS1 after the washes required by these methods. Moreover, we saw variable plating efficiency of L. discophora SS1 when transferred from liquid to solid medium even when cultures were transferred from the same stage of growth. This alone could account for the failure of Siering’s previous attempts (Siering, 1996).

DNA transfer by conjugation is a natural event that occurs frequently in the environment (Chen et al., 2005; Aminov, 2011). The minimal manipulation of cells required for this process eliminates some of the problems encountered with electroporation or chemical competency; we therefore reasoned that it would increase the probability of DNA transfer. Incompatibilities between the restriction systems of the donor and recipient are largely bypassed during conjugation as transferred DNA enters the recipient as a single strand and is methylated following second-strand synthesis, thus avoiding deleterious endonuclease attack (Šostková & Horaková, 1998). Also, whilst the size of the transferred DNA can be a limitation for electroporation, it does not affect conjugation as large vectors and genomes have been transferred successfully (Adelberg & Pittard, 1965).

After several failed attempts at conjugation between L. discophora SS1 and E. coli, the first successful experiment yielded only a single transconjugant. Whilst this proved to us that conjugation was possible, the development of a functional genetic system required much higher levels of

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**Fig. 1.** Schematic representation of pyrF disruption by integration of the plasmid pJSpyrF into the chromosome of L. discophora SS1 by homologous recombination. (a) An internal fragment of the pyrF gene was cloned in to the multiple cloning site (MCS) of plasmid pVIK165 to generate pJSpyrF, which also harbours neo encoding kanamycin resistance. (b) pJSpyrF integrated into the chromosome via recombination with the cloned pyrF fragment. (c) Interruption of the chromosomal copy of pyrF resulted in two non-functional pyrF fragments.
Conjugation and prompted the testing of a number of parameters to enhance the efficiency of plasmid transfer.

**Donor/recipient ratio**

The donor/recipient ratio is often the factor that is tested first in efforts to improve transfer efficiency (Fernandez-Astorga et al., 1992; Lampkowska et al., 2008). We found that ratios around 1:1 E. coli donor/recipient yielded the highest rates of conjugation for L. discophora SS1, which ranged from 0.5 to 3.1 × 10^2 transconjugants per donor (Table 4). The variability observed was probably due, at least in part, to the variable plating efficiency of L. discophora after transfer to solid medium. The efficiency of conjugation dropped rapidly when L. discophora SS1 was in greater abundance; conjugation efficiency decreased to ~10^2 transconjugants per donor when L. discophora SS1 was 10 times more abundant, with no transconjugants observed when the ratio favoured L. discophora SS1 at 100:1. This pattern is not particularly surprising, as it is hard to imagine how increasing the number of recipients whilst maintaining the number of donors could increase the number of transconjugants unless secondary transfer between recipients was occurring. The failure of increased donor numbers, which should have increased the possibility of cell-to-cell contact and hence the transfer of plasmid DNA from donor to recipient, was unexpected. The decrease in efficiency was less dramatic when the ratio changed in favour of the donor: we saw 30 times fewer transconjugants for donor/recipient ratios of 12:1 and 500 times fewer transconjugants for ratios of 50:1. Even fewer transconjugants per donor (10^-5) were obtained at ratios of 1000:1.

Optimal conjugation efficiency at donor/recipient ratios of ~1:1 is consistent with the results from other bacteria undergoing conjugation with E. coli (Schultheiss & Schüler, 2003), although in some cases conjugation has been reported to be more efficient when the donor is in excess. For example, donor/recipient ratios of 10^5:1 yielded the best efficiency for transfer to Bifidobacterium strains (Dominguez & O’Sullivan, 2012). Another study, however, found that results varied depending on the kind of donor, recipient and mobile element used (Willetts & Wilkins, 1984).

**Growth stage of recipient**

The impetus for testing this factor was the assumption that cells harvested at different growth stages would have different susceptibilities to conjugation. Conjugation is an energetic expense for both the donor and the recipient. We hypothesized that exponentially growing L. discophora SS1 cells would have more resources to spend on conjugation as opposed to cells in the stationary phase and that this difference would affect the number of transconjugants. Similar numbers of transconjugants were obtained for cells in the mid-exponential, late exponential and stationary phases, but these values were an order of magnitude higher than the number of transconjugants obtained with L. discophora SS1 from the early exponential phase (Table 5).

**Mating time**

The mating time has been reported to have an effect on the number of transconjugants for some bacteria, but not others (Chen et al., 2005). We wanted to determine a duration of mating for L. discophora SS1 that would allow sufficient time for plasmid transfer to occur, but not so long as to energetically deplete and stress the cells. Mating times of 6 and 12 h resulted in a similar number of

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**Fig. 2.** The growth pattern of a spontaneous nalidixic acid-resistant mutant, L. discophora SS1 Nal^+(•), was similar to that of the WT strain (○). Error bars represent SD of three biological replicates.
transconjugants, whereas the number was an order of magnitude lower for matings that lasted for 18 h (Table 6).

Other considerations

Neither recovery period nor the presence/absence of manganese had any effect on conjugation (Table 6).

Conjugation protocol

Based on the results obtained from testing the above parameters, a protocol was established that was used thereafter for routine conjugation of *L. discophora* SS1. Aliquots of 20 ml cultures grown in liquid medium to late exponential phase (OD_{600} =0.25) were harvested by centrifugation and mixed with the *E. coli* recipient in a 1:1 ratio, and then allowed to mate for 6–12 h on solid 2×PYG medium. The mating mixture was scraped from the plate, and then diluted and plated directly onto 2×PYG containing the appropriate antibiotics, without recovery. Although it had no effect on conjugation, manganese was included because it provided a rapid secondary confirmation that the transconjugants were *L. discophora* SS1. It was imperative that two or three dilutions of the mating mixture be plated, as a very dense or dilute mating mixture often did not yield any transconjugants. Dilutions in the range of $10^{-3}$ to $10^{-2}$ typically resulted in tens to hundreds of well-separated transconjugant colonies per plate, respectively. This observation might also help explain previous unsuccessful attempts to develop a genetic system for *L. discophora* SS1.

In general, colonies of transconjugants receiving the replicative plasmid appeared after 6–7 days, whilst transconjugants receiving the suicide plasmid only became visible after 10–12 days. Importantly, whilst transconjugants were obtained from most of the conjugations, it was difficult to obtain similar numbers of transconjugants even from identical replicates of the same experiments inoculated from the same culture. This suggested that there were variations within each culture of *L. discophora* SS1 that affected the viability and readiness of cells to undergo conjugation. The variability of *L. discophora* SS1 growth has been noted previously (El Gheriany, 2010).

Plasmid stability in *L. discophora* SS1

Some plasmids can be maintained stably in a host even without selective pressure, whilst other plasmids are easily lost once the selective pressure is removed (Coppi et al., 2001). The stability or instability of a plasmid in a host can therefore be used to advantage when developing genetic systems that rely on recombination, so long as they contain appropriate selection and counter-selection markers. We assessed the stability of the broad-host range vector pBBR1MCS-2 (Kovach et al., 1995). After six transfers and ~100 generations, 20 % of the cells that grew on non-selective media also grew on kanamycin plates. The loss of pBBR1MCS-2 at ~1 % per generation meant it was unlikely to be suitable for chromosomal integrations unless it was engineered to contain a negative selection marker (Fig. 3). By comparison, the same plasmid experienced 100 % loss within 12 generations in a *Geobacter sulfurreducens* host.

### Table 5.

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Frequency of transfer</th>
</tr>
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<tbody>
<tr>
<td>Early exponential phase (9 and 13 h)</td>
<td>$3.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Mid exponential phase (19 and 25 h)</td>
<td>$3.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>Late exponential phase (32 h)</td>
<td>$2.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>Stationary phase (44 h)</td>
<td>$3.7 \times 10^{-3}$</td>
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### Table 6.

<table>
<thead>
<tr>
<th>Mating time (h)</th>
<th>No recovery after mating</th>
<th>Recovery after mating</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No manganese</td>
<td>Manganese</td>
</tr>
<tr>
<td>6</td>
<td>$1.4 \times 10^{-5}$</td>
<td>$1.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>12</td>
<td>$1.9 \times 10^{-5}$</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>18</td>
<td>$5 \times 10^{-6}$</td>
<td>$5.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Identification of a potential gene to disrupt. To demonstrate the efficiency of the genetic system we developed for *L. discophora* SS1, we disrupted *pyrF*, which is predicted to encode orotidine 5′-phosphate decarboxylase, an enzyme involved in the *de novo* synthesis of pyrimidines (Haugaard & West, 2002). Inactivation of *pyrF* is known to result in resistance to 5-FOA and uracil auxotrophy (Fig. 4) (Haugaard & West, 2002; Galvão & de Lorenzo, 2005; Schneider et al., 2005). As expected, chromosomal integration of pJSpyrF into *L. discophora* SS1 led to 5-FOA resistance and uracil auxotrophy (Fig. 5a, b). When a WT copy of the gene was provided *in trans* on pBBR1MCS (a chloramphenicol-resistant derivative of pBBR1MCS2), the activity was restored (Fig. 5a, b).

This gene was chosen because it lends itself to both positive and negative selection strategies. Its orthologue (URA3) has been used widely in yeast genetic systems and more recently in bacteria, especially to create markerless mutations in strains with multiple natural antibiotic resistance determinants (Galvão & de Lorenzo, 2005; Schneider et al., 2005). *pyrF* codes for the last of the five enzymes found in the pathway for *de novo* production of pyrimidines (Fig. 4). Under normal circumstances, PyrE orthologues (orotate phosphoribosyltransferase) convert orotate to orotidine monophosphate (OMP), which is further transformed to uridine monophosphate (UMP) by PyrF. UMP is the precursor of all pyrimidine nucleotides.

When 5-FOA is added to the medium, it is converted to 5-F-OMP by PyrE and then to 5-F-UMP by PyrF. Whilst 5-FOA is not directly toxic itself, the accumulation of 5-F-UMP leads to inhibition of macromolecular synthesis (RNA) and cell death. Disruption of *pyrF* abolishes the activity of orotidine 5′-phosphate decarboxylase, preventing the production of toxic 5-F-UMP, essentially rendering cells resistant to 5-FOA. The other consequence of *pyrF* interruption is the loss of *de novo* uracil production, rendering the cells uracil auxotrophs. This combination of resistance/sensitivity to 5-FOA and uracil auxotrophy/prototrophy allows this strategy to be employed as both positive and negative selection (Takeno et al., 2004).

Beyond lending itself to both positive and negative selection, we chose to disrupt *pyrF* for several additional reasons. (i) In searching the draft genome of the closely related strain *L. cholognidi* SP-6 (GenBank accession number NC_010524.1), we identified a putative *pyrF* homologue whose upstream and downstream regions contained ORFs similar to the other four genes specific for *de novo* pyrimidine synthesis (*pyrB*, *pyrC*, *pyrD* and *pyrE*). (ii) We were able to isolate spontaneous 5-FOA-resistant *L. discophora* SS1 mutants (data not shown); we thus had preliminary evidence that this was an efficient selection system that resulted in an easily identified and tested phenotype. (iii) *pyrF* has potential to be developed into a more refined genetic tool allowing for the construction of markerless second deletions in a marked first-deletion background. (iv) *L. discophora* SS1 could not grow in 5% sucrose, which is required for counter-selection in the widely used *sacB* system (Kaniga et al., 1991).

**Gene disruption and in trans complementation.** After mating, hundreds of *L. discophora* SS1 transconjugants were obtained on both nalidixic acid/kanamycin and nalidixic acid/kanamycin/5-FOA, whilst only a few colonies were obtained on nalidixic acid/5-FOA, suggesting that the majority of 5-FOA-resistant mutants arose due to insertion of the plasmid at the *pyrF* locus, and not to the acquisition of spontaneous resistance to 5-FOA and kanamycin.

PCR was used to confirm single-crossover integration of the 5 kb plasmid at the *pyrF* locus (Fig. 1). Whilst the internal *pyrF* fragment could be amplified from both the WT *L. discophora* SS1 and the *pyrF* mutant, the full gene could not be amplified from the SS1:pJSpyrF mutant. When the extension time was increased to 3 min, a PCR product of ~5 kb resulted, consistent with the amplification of the entire plasmid that disrupted *pyrF* (data not shown). A primer pair designed to anneal to the region upstream of *pyrF* and to the GFP-encoding gene on the plasmid only amplified a product from the integration mutant and not the WT, confirming the presence of the plasmid in the chromosome at the *pyrF* locus.

![Fig. 3. Loss of replicative plasmid pB1MCS-2 from L. discophora SS1 cells in the absence of antibiotic selection. Around 80% of the cells (□) lost the plasmid over >100 generations (seven transfers over 18 days) when grown without antibiotic as compared with the same culture grown in the presence of antibiotic (▲).](https://www.microbiologyresearch.org)
To confirm that the resistance to 5-FOA was a result of disrupting the activity of orotidine 5′-phosphate decarboxylase (PyrF) and not to unexpected secondary mutations or some unanticipated effect on downstream gene expression, a WT copy of *pyrF* with its native promoter was provided on plasmid pBBR1MCS (pBBpyrF). An *in silico* search of the upstream region of *pyrF* for its native promoter revealed that the gene *purB* (involved in the synthesis of purines) was located only 11 nt upstream of *pyrF* and that the nearest easily identifiable promoter was upstream of *purB*. Although *purB* should not be required for complementation, pBBpyrF included *purB* and its promoter as well as *pyrF* because efforts to artificially clone the *purB* promoter immediately adjacent to *pyrF* failed despite numerous attempts.

*L. discophora* SS1 Nat⁺ : pJSpyrF was transformed with pBBpyrF and transconjugants were readily obtained on nalidixic acid, kanamycin and chloramphenicol. As seen in Fig. 5(a), the complemented strain behaved in a similar manner to WT *L. discophora* SS1 and could not grow in medium with 5-FOA. In contrast, 5-FOA did not have a toxic effect on *L. discophora* SS1 Nat⁺ : pJSpyrF with or without the vector control. When assessed for uracil auxotrophy, the *pyrF* mutant with or without the empty vector could not grow in medium without uracil, confirming a loss of *de novo* uracil production by this mutant. As expected, growth of the mutant was restored to near-WT levels when uracil was provided in the medium (Fig. 5b). Again, the complemented mutant showed WT uracil prototrophy and 5-FOA sensitivity, providing clear evidence of complementation. Overall, these assays confirmed that the genetic system described here enabled successful manipulation of a gene of interest in *L. discophora* SS1.

This work paves the way for the creation of a clean deletion of *pyrF* by flanking the *neo* gene on pVIK165 with fragments up and downstream of *pyrF*, and selecting for a second crossover on 5-FOA. Mutagenizing a Δ*pyrF* mutant with a suicide vector containing a WT copy of *pyrF* as the counter-selectable marker and a chromosomal fragment targeted for insertion (e.g. *mofA*) would allow for selection of transconjugants into other chromosomal locations. A single crossover of the suicide plasmid into *mofA* or other locations would provide a copy of *pyrF* and restore *de novo* uracil biosynthesis and growth on minimal medium. A second crossover resulting in a clean deletion of *mofA* could be selected for by exposure to 5-FOA and the accompanying loss of the plasmid-borne *pyrF*. In theory, such a system could be used again and again to generate multiple markerless mutations in the same background, as has been described for *pyrF*-based systems in other organisms (Haugaard & West, 2002; Takeno *et al.*, 2004; Galvão & de Lorenzo, 2005; Schneider *et al.*, 2005).

**CONCLUSION**

We have shown that plasmid DNA can be introduced into *L. discophora* SS1 by conjugation at high transfer frequencies that are comparable to other microbial systems...
and enable further genetic manipulations such as isolation of chromosomal integrants. Most importantly, we were able for the first time, to our knowledge, to develop a genetic system for *L. discophora* SS1. We confirmed its effectiveness by inactivating a gene of interest (*pyrF*) followed by complementation in trans with a plasmid-borne copy of *pyrF*. Together, these techniques should help us identify previously uncharacterized genes that may have a role in *L. discophora* SS1 physiology, including manganese oxidation.

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