INTRODUCTION

*Lactococcus garvieae* is the aetiological agent of lactococcosis, an emergent disease, which affects cultured freshwater and marine fish with special incidence in rainbow trout (*Oncorhynchus mykiss*) (Eldar *et al.*, 1999a) and yellowtail (*Seriola quinqueradiata*) (Kusuda & Kawai, 1998), particularly during the summer given its association with high water temperatures (for a review see Vendrell, 2006). In addition, *L. garvieae* has been isolated from buffalos with mastitis (Teixeira *et al.*, 1996), from clinical specimens of human blood and urine (Elliott *et al.*, 1991) and from patients with bacterial endocarditis and different tissue infections (Aguirre & Collins, 1993; Fefer *et al.*, 1998; James *et al.*, 2000; Mofredj *et al.*, 2000; Fahman *et al.*, 2006; Vinh *et al.*, 2006; Wang *et al.*, 2006; Yiu *et al.*, 2007).

In fish farming, outbreaks are treated with antibiotics, although they are often ineffective and do not prevent reinfection. On the other hand, vaccination with inactivated whole cells by intraperitoneal injection is only protective for a limited period of time (Ravelo *et al.*, 2005). In recent years, progress has been made in diagnostic techniques (Endo *et al.*, 1998; Zlotkin *et al.*, 1998; Goh *et al.*, 2000; Wilson & Carson, 2003), strain typing (Eldar *et al.*, 1999b; Vela *et al.*, 2000; Wilson *et al.*, 2002; Ravelo *et al.*, 2003; Barnes & Ellis, 2004; Eyngor *et al.*, 2004; Kawanishi *et al.*, 2006) and the knowledge about the immune response to infection (Ooyama *et al.*, 1999; Barnes *et al.*, 2002b; Schmidtke & Carson, 2003; Shin *et al.*, 2007). Despite the importance of this syndrome, there is little information about the precise pathogenic mechanisms by which this bacterium is able to defeat the host defences and cause disease. Up to now, it has only been established that virulence of this bacterium is, in part, dependent upon its ability to form a capsule (Yoshida *et al.*, 1997; Barnes *et al.*, 2002a).

Laboratory media appear to be a limited tool when trying to study the molecular mechanisms of disease, since it is very difficult to mimic the complex and changing environment of the host. In an attempt to overcome these limitations, Hensel *et al.* (1995) developed a method for

Genes required for *Lactococcus garvieae* survival in a fish host

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*Lactococcus garvieae* is considered an emergent pathogen in aquaculture and it is also associated with mastitis in domestic animals as well as human endocarditis and septicemia. In spite of this, the pathogenic mechanisms of this bacterium are poorly understood. Signature-tagged mutagenesis was used to identify virulence factors and to establish the basis of pathogen–host interactions. A library of 1250 *L. garvieae* UNIUD074-tagged Tn917 mutants in 25 pools was screened for the ability to grow in fish. Among them, 29 mutants (approx. 2.4 %) were identified which could not be recovered from rainbow trout following infection. Sequence analysis of the tagged Tn917 interrupted genes in these mutants indicated the participation in pathogenesis of the transcriptional regulatory proteins homologous to GidA and MerR; the metabolic enzymes asparagine synthetase A and α-acetolactate synthase; the ABC transport system of glutamine and a calcium-transporting ATPase; the dltA locus involved in alanylation of teichoic acids; and hypothetical proteins containing EAL and Eis domains, among others. Competence index experiments in several of the selected mutants confirmed the relevance of the Tn917 interrupted genes in the development of the infection process. The results suggested some of the metabolic routes and enzymic systems necessary for the complete virulence of this bacterium. This work is believed to represent the first report of a genome-wide scan for virulence factors in *L. garvieae*. The identified genes will further our understanding of the pathogenesis of *L. garvieae* infections and may provide targets for intervention or lead to the development of novel therapies.

Abbreviations: CI, competitive index; STM, signature-tagged mutagenesis.

The GenBank/EMBL/DDBJ accession numbers for the DNA sequences corresponding to mutants I–XXVI are EF450028–EF450053, respectively.
the identification of bacterial virulence genes by screening in living animals, called signature-tagged mutagenesis (STM). STM relies on two key elements. First, a negative selection will select against replication of mutant strains where a transposon-mediated disruption of genes related to virulence has occurred. Second, as each mutagenizing transposon carries ‘signature tags’ (DNA fragments consisting of a central variable region that is flanked by two invariant arms to which oligoprimers may bind for PCR amplification), STM is capable of distinguishing between the different mutants. A limitation of the application of STM is that the screen will be unlikely to detect mutations in virulence determinants whose function can be trans-complemented by the presence of other strains, i.e. toxins, adhesins and binding proteins. Conversely, it may favour the identification of genes having a longer-term impact on in vivo growth and persistence. The STM methodology has been adapted and used to identify virulence genes in several microbial pathogens (Saenz & Dehio, 2005). Modifications of this method include the use of pre-selected tagged transposons to construct the mutant library, which simplifies the screening significantly (Mei et al., 1997). This modification was used in the present work.

In this study, STM was used to identify genes required for growth and survival in a fish model of infection. A library of 1250 L. garvieae UNIUD074-tagged Tn917 mutants in 25 pools was screened for attenuation. This led to the identification of 29 mutants defective for survival in the animal host, as judged by their inability to compete with the wild-type strain in mixed infections. Thus, this work establishes a first approach to the study of the genes that are required for survival of L. garvieae in rainbow trout.

METHODS

Bacterial strains, plasmids and culture conditions. L. garvieae strain UNIUD074 was obtained from Dr L. Gusmani (University of Udine, Italy). Plasmid pTV408 (Slater et al., 2003) was obtained from Dr J. P. May (Department of Clinical Veterinary Medicine, University of Cambridge, UK). L. garvieae strains were routinely cultured in brain heart infusion medium (BHI) (Difco) at 20, 28 or 40 °C. Escherichia coli strain DH5αpir was grown in 2 × TY (tryptone/yeast) medium at 37 °C. Two per cent agar was added to obtain solid media. The following antibiotics were added to the media as needed: 1 µg erythromycin ml⁻¹ for L. garvieae and 100 µg ampicillin ml⁻¹ for E. coli.

DNA manipulations, PCR, digoxygenin labelling and hybridizations. Extraction of chromosomal and plasmid DNA from L. garvieae was performed as described by Leenhouts et al. (1989). Plasmid DNA from E. coli was prepared by alkaline lysis (Birnboim & Doly, 1979). Routine DNA manipulation was conducted as described by Sambrook & Russell (2001). Phage T4 DNA ligase and calf intestinal alkaline phosphatase were from Amersham, and oligonucleotides were from Sigma.

The tag region was amplified by PCR using the primers P2 (5'-ATTCTACAAACCTCAGC-3') and P4 (5'-ATTCACTTCAAACAGC-3') (Hensel et al., 1995). Synthesis of digoxygenin (DIG)-labelled tags, hybridization and development were performed with a DIG DNA labelling and detection kit according to the manufacturer’s instructions (Roche Applied Science). For dot-blot hybridizations, plasmid DNA (1 µg) was transferred onto a Hybond-N⁺ membrane (Amersham) using a Bio-Dot Microfiltration Apparatus (Bio-Rad). DNA on the membranes was denatured by alkali treatment and fixed by UV cross-linking according to the manufacturer’s instructions.

Southern hybridization analysis was performed by standard methods on EcoRI-digested genomic DNA using an 819 bp fragment from the bla gene present in the transposon Tn917 as a probe (Menendez et al., 2006).

Cloning and selection of tags. pTV408 is a thermo-sensitive plasmid able to replicate at temperatures below 37 °C but not above this temperature (Slater et al., 2003). It harbours the Tn917 transposon conferring erythromycin resistance. A single EcoRI site is present in the plasmid and it is located within the Tn917 transposon. This EcoRI site was used to introduce the PCR-generated tags. Double-stranded 89 bp DNA signature tags were obtained by PCR using the variable oligonucleotide pool RT (5'-CTAGAATTCTACAAACCTCAGC-3'-NK) and P3 (5'-CATGAAATTCTCAACCT-3'), which are the same as primers P5 and P3 (Hensel et al., 1995) except that the 5' ends have a site for EcoRI instead of PstI. The PCR-amplified tags were digested with EcoRI and gel purified, then ligated with EcoRI-digested, dephosphorylated pTV408 to generate pTV408tag. The ligated DNA was transformed into L. garvieae UNIUD074 by electroporation (Menendez et al., 2006).

Transformed bacteria were plated onto BHI containing erythromycin and grown at 28 °C overnight. The total pool of transformants was then screened by colony blot hybridization with their corresponding DIG-labelled tags to identify 50 tags that amplified and labelled efficiently. These tags were then tested for cross-hybridization (Hensel et al., 1995) and 50 transformants were chosen for library construction.

Generation of the L. garvieae mutant bank. A single colony of each of the transformants chosen was transferred into a microtitre dish well containing BHI broth and erythromycin. The microtitre dish was incubated at 28 °C overnight. Glycerol was added to each well to give a final concentration of 50% (v/v), and the plate (designated the master plate) was stored at −80 °C.

To generate 50 different Tn917 mutants, bacteria from the master plate were replicated using a microtitre dish replicator (Sigma) into the wells of a second microtitre dish containing 200 µl BHI broth and erythromycin. This dish was incubated at 40 °C overnight, and then bacteria from each well were streaked onto BHI agar containing erythromycin and incubated at 40 °C to obtain single colonies. One different colony from each well was then transferred into the corresponding well of a microtitre dish containing BHI broth and erythromycin. Mutant pools were also stored at −80 °C in 50% (v/v) glycerol.

A series of identical membranes for dot-blot hybridizations was prepared by transferring 1 µg of each of the selected 50 plasmids onto Hybond-N⁺ membranes using the Bio-dot Microfiltration apparatus.

In vivo mutant selection. Each pool of 50 mutants was grown in BHI supplemented with erythromycin in a microtitre dish at 20 °C overnight. The bacteria were pooled, washed twice with PBS and resuspended in PBS. According to previous results obtained by LD₅₀ experiments using the parental strain, conditions for selection of mutants in vivo were defined (A. Menéndez & J. A. Guijarro, unpublished). Rainbow trout (O. mykiss) weighing from 10 to 15 g were infected by intraperitoneal injection (for screening the library) with doses of 10⁶ mutant cells in 100 µl PBS. Fish were kept in 60 l tanks at 20 °C in continually flowing dechlorinated water. At 72 h
post-infection, the animals were sacrificed and dissected, and the liver and spleen were homogenized in BHI with a stomacher. Afterwards, the pool of bacteria was grown in BHI supplemented with erythromycin, and chromosomal DNA was isolated for the generation of the output PCR probe (Hensel et al., 1995). The tags present in the recovered pools were compared with the tags present in the inoculum pools by PCR amplification from DNA samples, DIG labelling using primers P2 and P4 and hybridizations to the 50 plasmids on Hybond-N⁺ membranes.

Characterization of transposon insertion sites. For plasmid rescue of mutants with attenuated virulence, genomic DNA previously digested with EcoRI was religated and the ligation mixture was used for transforming E. coli DH5αpir electrocompetent cells. Transformants were selected on 2×TY agar plates containing 100 μg ampicillin ml⁻¹. Plasmid DNA was obtained from the transformants and sequencing analysis was performed, as described by Slater et al. (2003), using the Tn917 seq (5'-AGAGAGATCTCAGGCTCAAAT-3') designed to read out from the transposon. The dideoxy chain-termination method was used for DNA sequencing using a DR terminator FS sequencing kit (Applied Biosystems). The sequence was obtained in an ABI Prism 310A automated DNA sequencer from Perkin-Elmer, according to the manufacturer’s instructions, at the Universidad de Oviedo facility. Sequence analysis was performed using the BLASTX computer program.

Competition experiments. For in vivo competition assays, mutant strains and the wild-type strain UNIUD074 were grown separately in BHI at 20°C for approximately 18 h. Bacteria were washed in PBS as described above, and each mutant was mixed with the wild-type at a concentration of 10⁶ c.f.u. ml⁻¹ each (2×10⁹ c.f.u. total bacteria ml⁻¹). Dilutions of this suspension were plated onto BHI (to measure total c.f.u.) and BHI with erythromycin (to determine mutant c.f.u.). From this, the exact input ratio of mutant to wild-type was calculated. A sample of 0.1 ml of the mixed suspension was used to infect rainbow trout weighing from 10 to 15 g by intraperitoneal injection. After 72 h, spleens and livers were recovered as described above, and homogenates were plated onto selective media to determine the output ratio of mutant to wild-type. The competitive index (CI) is defined as the output ratio (mutant/wild-type) divided by the input ratio (mutant/wild-type).

For in vitro competition assays, 5 ml BHI in a test tube was inoculated with approximately 10⁵ c.f.u. ml⁻¹ of the mutant and the wild-type. The cultures were grown at 20°C for 18 h (final OD₆₀₀ was 1.5–2). The input and output ratios of mutant to wild-type were determined by selective plating as described above.

RESULTS AND DISCUSSION

Construction of signature-tagged L. garvieae mutant library

From the original pool of signature-tagged transposons, 50 uniquely tagged transposons were selected and used to construct the mutant library. The criteria of selection were efficient amplification and labelling, and lack of cross-hybridization to other tags. The plate containing this array of 50 transformants was designated the master plate and was used for all subsequent mutagenesis of L. garvieae. To generate 50 different Tn917 mutants, bacteria from the master plate were replicated into the wells of a second microtitre dish, grown at the non-permissive temperature (40°C) and then mutant bacteria from each well were purified and transferred into a third microtitre dish, as described in Methods. Thus, a total of 1250 mutants was arrayed in 25 pools of 50 mutants each.

Screening for essential genes for infection in fish

For the in vivo selection of attenuated strains, a total of 1250 different tagged mutants were assembled into 25 pools of 50 different mutants. The presence of attenuated strains in each pool was analysed by comparison between the strains that were inoculated (input) to those that were recovered from the livers and spleens of the animals (output). Failure of recovery from the output was considered as possible attenuation. Output pools were recovered 72 h post-infection because intraperitoneal experimental infection in rainbow trout caused the first symptoms at that time after inoculation. Bacterial doses were defined by previous LD₅₀ experiments using the parental strain (A. Menéndez & J. A. Guijarro, unpublished). Mutants were identified as attenuated if they showed a reproducible decrease in the hybridization signal between input and output pools in two animals. An example of the results of a hybridization analysis is shown in Fig. 1. Hybridization signals at positions B1, B3, C6 and E1 are weaker on the blots probed with tags from the recovered pools (output pools) than on the blot probed with tags from the inoculum pool (input pool). Twenty-nine putative attenuated mutants were identified in the STM screening of 1250 mutants (approx. 2.4%). To verify that selected mutants carried a single chromosomal insertion of Tn917, chromosomal DNA samples from the individual mutant strains were digested with EcoRI and subjected to Southern analysis using part of the bla gene as a probe. For each of the analysed mutants, a single hybridizing fragment of different size was observed in each lane. This indicates that Tn917 insertions occurred singly in the chromosome of the L. garvieae mutants (data not shown).

Identification of disrupted genes

The DNA regions flanking the pTV408tag side of the transposon insertion points of 29 mutants were cloned as described in Methods. The nucleotide sequences of the flanking regions were determined and subsequently analysed by searching the DNA and protein databases for identical or similar genes (Table 1). Twenty mutants had Tn917 flanking DNA encoding proteins with identities to known proteins. Transcriptional products of flanking DNA cloned from four mutants did not display any similarity to sequences in the public databases. Finally, another five mutants showed identities with proteins of unknown function.

Unexpectedly, four out of the 20 mutants in genes encoding known proteins had transposon insertions in different locations within a 1.7 kb DNA genomic region containing two genes (asmA and merR). Random Tn917 transposon insertion throughout the chromosome of Streptococcus mutans (Gutierrez et al., 1996), group A
### Table 1. Characteristics of the genes identified by STM screens with fish

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Homology</th>
<th>Hypothesized function</th>
<th>% Amino acid identity (homology)</th>
<th>CI in vitro</th>
<th>CI in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>AsnA/Clostridium perfringens</td>
<td>Asparagine synthetase</td>
<td>57 (74)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>GidC/Lactococcus lactis subsp. lactis</td>
<td>Enzyme possibly involved in translation</td>
<td>91 (95)</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>III</td>
<td>ChiA/L. lactis subsp. cremoris</td>
<td>Chitinase</td>
<td>52 (65)</td>
<td>0.75</td>
<td>0.09</td>
</tr>
<tr>
<td>IV</td>
<td>Als/L. lactis subsp. lactis</td>
<td>α-Acetolactate synthase</td>
<td>82 (92)</td>
<td>0.45</td>
<td>0.14</td>
</tr>
<tr>
<td>V</td>
<td>Hypothetical protein/Pediococcus pentosaceus</td>
<td>Unknown</td>
<td>51 (78)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VI</td>
<td>MerR/Enterococcus faecium</td>
<td>Transcriptional regulator</td>
<td>59 (83)</td>
<td>0.93</td>
<td>0.03</td>
</tr>
<tr>
<td>VII</td>
<td>Xre/L. lactis subsp. cremoris</td>
<td>Transcriptional regulator</td>
<td>33 (59)</td>
<td>0.91</td>
<td>0.95</td>
</tr>
<tr>
<td>VIII</td>
<td>AsnA/Lactobacillus salivarius subsp. salivarius</td>
<td>Asparagine synthetase</td>
<td>28 (38)</td>
<td>0.87</td>
<td>0.2</td>
</tr>
<tr>
<td>IX</td>
<td>Hypothetical protein/Staphylococcus aureus subsp. aureus</td>
<td>Unknown</td>
<td>100 (100)</td>
<td>0.4</td>
<td>&lt;0.0006</td>
</tr>
<tr>
<td>X</td>
<td>Yrb/I. lactis subsp. lactis II1403</td>
<td>Transcriptional regulator</td>
<td>34 (59)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XI</td>
<td>Unknown protein/Enterococcus faecalis</td>
<td>Unknown</td>
<td>46 (67)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XII</td>
<td>No homology in public databases</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XIII</td>
<td>Calcium-transporting ATPase/Ent. faecium</td>
<td>Inorganic ion transport</td>
<td>49 (66)</td>
<td>0.85</td>
<td>0.11</td>
</tr>
<tr>
<td>XIV</td>
<td>No homology in public databases</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XV</td>
<td>No homology in public databases</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XVI</td>
<td>Hypothetical protein/Bacillus cereus</td>
<td>Unknown</td>
<td>50 (66)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XVII</td>
<td>XerC/Straptooccus pneumoniae</td>
<td>Integrase/recombinase</td>
<td>62 (78)</td>
<td>0.79</td>
<td>0.34</td>
</tr>
<tr>
<td>XVIII</td>
<td>DltA/L. lactis subsp. lactis II1403</td>
<td>d-Alanine-d-alanyl carrier protein ligase</td>
<td>68 (74)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XIX</td>
<td>GlnP/L. lactis subsp. lactis II1403</td>
<td>ABC transporter glutamine</td>
<td>78 (90)</td>
<td>0.42</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>XX</td>
<td>No homology in public databases</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XXI</td>
<td>DprA/L. lactis subsp. lactis</td>
<td>DNA processing SMF protein</td>
<td>58 (85)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XXII</td>
<td>Hypothetical protein/Lactobacillus gasseri</td>
<td>RNA polymerase binding protein (family Spx)</td>
<td>37 (69)</td>
<td>1.5</td>
<td>0.46</td>
</tr>
<tr>
<td>XXIII</td>
<td>Hypothetical protein/Ent. faecium</td>
<td>Unknown</td>
<td>32 (46)</td>
<td>0.64</td>
<td>0.4</td>
</tr>
<tr>
<td>XXIV</td>
<td>Hypothetical protein/L. lactis</td>
<td>Protein with an EAL domain</td>
<td>37 (58)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XXV</td>
<td>Hypothetical protein/L. lactis subsp. cremoris SK11</td>
<td>Acetyltransferase involved in intracellular survival</td>
<td>52 (69)</td>
<td>1.25</td>
<td>0.01</td>
</tr>
<tr>
<td>XXVI</td>
<td>Intergenic region between asnA and merR</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

–, No data.
streptococci (Eichenbaum & Scott, 1997) and Streptococcus equi (Slater et al., 2003) has been assumed based on the appearance of different-sized hybridizing fragments on Southern blots. However, caution must be used when interpreting these results because, as Slater et al. (2003) demonstrated, when the insertions were analysed using the Strep. equi genome sequence database, 60% of the transposition insertions in this bacterium were found within a 15 kb region of its genome, whereas the remaining 40% appeared to be random (Slater et al., 2003). Previous studies by Southern blot analysis of Tn917 insertion into the L. garvieae genome suggested that this occurred apparently randomly (Menendez et al., 2006). Nevertheless, the selection in the present work of seven mutants that had transposon insertions in four different locations within a DNA region of 1.7 kb suggests that a hot-spot for Tn917 insertion exists in the chromosome of L. garvieae. This is consistent with the idea that all transposons exhibit some sequence bias for transposition (Coulter et al., 1998).

**Competitive assays with selected mutants**

*In vivo* competition assays were performed to validate the results of our STM screen and to quantify the degree of virulence attenuation of individual mutants. In the assay, mixed infections with mutant and wild-type strains are used to provide an *in vivo* measure of virulence attenuation referred to as the *in vivo* CI (see Methods). A total of 13 of the 24 different mutants were selected. The mutants displayed a range of attenuation in the *in vivo* competitive assays (Table 1), ranging from subtle attenuation (XXII, CI=0.46) to severe attenuation (IX, CI<0.0006). Mutant VII was not attenuated in the *in vivo* competition assay (Table 1). This mutant represents a false-positive mutant, a result that has also been seen in other screens (Camacho et al., 1999; Ruley et al., 2004). In addition, comparable general growth defects under optimal laboratory growth conditions were ruled out by *in vitro* competition assays (see Methods). Thus, three mutants (IV, IX and XIX) showed a growth defect, whereas the rest of the analysed mutants showed essentially wild-type growth (Table 1).

**Inferred function of STM selected genes**

Approximately 70% of the mutants identified had transposon insertions within genes encoding proteins similar to known proteins in the public databases.

The ability to adapt to the host environment is a key component of pathogenicity. The nutritional environment of the host’s cells imposes a requirement for *de novo* biosynthesis of various amino acids, cofactors and nucleotides in many pathogens. The identification of mutants with transposon insertions that induce auxotrophy demonstrated that auxotrophic mutants are cleared from the host. Two mutants (I, VIII) were found with transposon insertions in different positions of an asparagine synthetase (AsnA) gene homologue that seems to be necessary for the survival of L. garvieae in the host.

Mutant IV had a transposon insertion in an *α*-acetolactate synthase gene homologue. This enzyme is involved in the biosynthesis of acetoin and 2,3-butanediol. The metabolic function of this process appears to be to counteract lethal acidification as cells approach stationary phase by redirecting pyruvate into neutral rather than acidic end products. The 2,3-butanediol pathway may also participate in the regulation of the NAD/NADH ratio in bacteria (Johansen et al., 1975). Recently, Yoon & Mekalanos (2006) obtained evidence that 2,3-butanediol synthesis gives *Vibrio cholerae* El Tor biotypes a survival advantage during infection, which is important for colonization. The results obtained in L. garvieae indicate the importance of the 2,3-butanediol pathway for *in vivo* survival.

A number of loci encoding putative regulatory genes were identified in the screening, suggesting the importance of regulation of bacterial gene expression for *in vivo* survival of L. garvieae. Mutant II had a transposon insertion in a homologue of the gidA gene. gidA is widely distributed and highly conserved in both prokaryotes and eukaryotes, having a translational regulatory function. (Kinscherf & Willis, 2002; Sha et al., 2004).

The DNA flanking the transposon insertion in mutant VI had homology to the regulatory protein MerR of *Enterococcus faecium*. The MerR family is a group of transcriptional activators that have been found in a wide range of bacterial genera. The majority of regulators in this family respond to environmental stimuli, such as oxidative stress, heavy metals or antibiotics (Amabile-Cuevas & Demple, 1991; Brown et al., 2001; Kim et al., 2002).

Mutant XXIV had a transposon insertion in a gene encoding a putative protein that contains an EAL domain. Proteins with this domain are predicted to regulate cell surface adhesiveness, biofilm formation and virulence in response to extracellular cues, by controlling the level of the newly recognized bacterial second messenger 3’,5’-cyclic diguanylic acid (c-diGMP) (Römling & Amikam, 2006). Genetic and biochemical evidence suggests that the EAL protein domains act as phosphodiesterase for c-diGMP degradation (D’Argenio & Miller, 2004). Recently, STM of *Salmonella* spp. found that the cdgR gene is required in order to resist the host phagocyte oxidase *in vivo*. CdgR consists solely of an EAL domain. Thus, besides its known role in regulating biofilm formation, bacterial c-diGMP also regulates host–pathogen interactions involving antioxidant defence and cytotoxicity (Hisert et al., 2005).

Other regulatory functions affecting *in vivo* survival were also identified, including a transcriptional regulator (Yrb1) from *Lactococcus lactis* (mutant X), and a putative RNA polymerase binding protein (mutant XXII). The assortment of independent mutations recovered in regulatory genes emphasizes the biological significance of these loci for *in vivo* survival of L. garvieae.
Two loci identified in the screening included homologues of transporter genes. DNA flanking the transposon insertion in mutant XIX showed homology to \( \text{glnP} \) of \( \text{L. lactis} \). The \textit{in vivo} attenuated growth seen in this mutant is probably caused by the polar effects of transposon insertion, as \( \text{glnP} \) and \( \text{glnQ} \) are usually organized in an operon. \( \text{glnP} \) encodes a glutamine ABC transporter permease and \( \text{glnQ} \) encodes a glutamine ABC transporter ATP-binding protein. It seems that the concentration of glutamine in the host is a critical parameter for \textit{in vivo} survival as occurred in group B streptococci (Tamura \textit{et al.}, 2002) and \textit{Streptococcus pneumoniae} (Polissi \textit{et al.}, 1998). Mutant XIII had a transposon insertion in a gene coding for a cation-transporting ATPase. A mutant in a hypothetical cation-transporting ATPase was also identified by STM in \textit{Strep. pneumoniae} (Polissi \textit{et al.}, 1998).

Other genes identified were associated with several cellular processes. Mutant XVII contained an insertion in a homologue to the tyrosine recombinase (XerC) of \textit{Strep. pneumoniae}. XerC has a role in the segregation of replicated chromosomes during cell division. The \( \text{xerC} \) null mutants of \textit{Strep. pneumoniae} and \textit{Staphylococcus aureus} were found to be attenuated in a murine infection model (Chalker \textit{et al.}, 2000). A site-specific recombinase was also found to be as important for survival of \textit{Erwinia amylovora} in plants (Wang & Beer, 2006). This result, together with that obtained in \textit{L. garvieae}, suggests that this gene may control processes affecting virulence.

Mutant XVIII had a transposon insertion in a gene encoding a protein homologous to DltA. This gene encodes a D-alanine-D-allyl carrier protein ligase involved in D-alanylation of teichoic acids in Gram-positive pathogens (Abachin \textit{et al.}, 2002; Weidenmaier \textit{et al.}, 2005; Kovács \textit{et al.}, 2006; Wartha \textit{et al.}, 2007). Interestingly, \( \text{dltA} \) mutants of \textit{Staph. aureus} (Weidenmaier \textit{et al.}, 2005), \textit{Strep. pneumoniae} (Wartha \textit{et al.}, 2007) and \textit{Listeria monocytogenes} (Abachin \textit{et al.}, 2002) showed attenuated virulence. In \textit{Strep. pneumoniae} the alanylation of teichoic acids is essential for protection against neutrophils during the infection process (Wartha \textit{et al.}, 2007). It seems that the \( \text{dltA} \) gene is important for growth of \textit{L. garvieae} in the host, but further studies are needed to elucidate the role of this gene in the \textit{L. garvieae} infection process.

Mutant XXI carried an insertion in the \( \text{dprA} \) gene, which has been described as a competence gene. The DprA protein has been suggested to be involved in the protection of incoming DNA. However, members of the \( \text{dprA} \) gene family (also called \( \text{smf} \)) can be detected in virtually all bacterial species, which suggests that their gene products have a more general function. The basic function of \( \text{dprA} / \text{smf} \) remains unclear (Smeets \textit{et al.}, 2006).

Mutant XXV had a transposon insertion in a gene coding for a putative protein that contains an Eis domain. The proteins with this domain are hypothetical and have unknown function. However, the Eis domain seems to be involved in intracellular survival. Although its function remains unknown, it was found to enhance intracellular survival of \textit{Mycobacterium smegmatis} in a human macrophage-like cell line when \( \text{eis} \) was introduced into \textit{M. smegmatis} on a multicopy vector (Wei \textit{et al.}, 2000). Intracellular survival may be essential for the progress of the infection by \textit{L. garvieae}. Additional studies are planned to further characterize this mutant and to determine the exact role of this protein.

The DNA flanking the transposon insertion in mutant III had homology to a chitinase of \textit{L. lactis}. The exact role of this protein during the infection is unknown. This mutant was severely attenuated in the CI assay \textit{in vivo} (Table 1). Further studies are under way to characterize this mutant.

Analysis of DNA sequences from nine mutants did not reveal any significant similarities to entries in the DNA and protein databases (XX, XII, XIV, XV) or reveal significant similarities to genes with unknown function (V, IX, XI, XVI, XXIII). CI assay analysis of one of the strains with mutations in genes with unknown function (XXIII) showed that it was significantly attenuated compared with the wild-type strain (Table 1).

**CONCLUSIONS**

The STM technique is a powerful method that allows large numbers of mutants to be screened for attenuation (competitive defects) in an animal model of infection. STM has been applied successfully to other Gram-positive bacterial pathogens including \textit{Strep. pneumoniae}, \textit{Staph. aureus} and \textit{Streptococcus agalactiae} (Polissi \textit{et al.}, 1998; Mei \textit{et al.}, 1997; Jones \textit{et al.}, 2000). The purpose of this study was to assess the usefulness of the technique for identifying virulence genes of \textit{L. garvieae}.

STM analyses of \textit{L. garvieae} identified a wide variety of functional gene classes underscoring the diversity of bacterial processes required for the infection process. Currently, the function of the gene identified in the screen can only be inferred by homology. Multiple mutants were obtained in genes homologous to transport systems, regulatory proteins and metabolism enzymes, suggesting the importance of their respective functions for infection. Mutants lacking \( \alpha \)-acetolactate synthase, teichoic acids alanylation protein (DltA), EAL and Eis proteins domains, and MerR and GidA regulatory proteins, seem to be interesting candidates for future studies. As in other STM studies, a relatively high percentage of the attenuated mutants were disrupted in genes of unknown function. These genes could potentially encode novel factors that may play important roles in the bacterial infection process. The identification of the entire ORF and search for specific motifs or domains may give clues about the putative function of these proteins. In addition, virulence studies of some of the mutants could give information about their potential for use as attenuated vaccines.
These results are believed to represent the first report of a genome-wide scan for virulence factors in *L. garvieae*, and a number of important putative virulence factor genes worthy of further study have been identified.

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