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### ► To cite this version:

P. Reimundo, A. Menéndez, J. Méndez, D. Pérez-Pascual, R. Navais, et al.. gene mutation in the teichoic acids alanylation system of results in diminished proliferation in its natural host.. *Veterinary Microbiology*, 2010, 143 (2-4), pp.434. 10.1016/j.vetmic.2009.12.004 . hal-00594816

**HAL Id: hal-00594816**

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Submitted on 21 May 2011

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## Accepted Manuscript

Title: *DltA* gene mutation in the teichoic acids alanylation system of *Lactococcus garvieae* results in diminished proliferation in its natural host.

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PII: S0378-1135(09)00595-1  
DOI: doi:10.1016/j.vetmic.2009.12.004  
Reference: VETMIC 4701

To appear in: *VETMIC*

Received date: 27-5-2009  
Revised date: 27-11-2009  
Accepted date: 2-12-2009

Please cite this article as: Reimundo, P., Menéndez, A., Méndez, J., Pérez-Pascual, D., Navais, R., Gómez, E., Braña, A.F., Guijarro, J.A., *DltA* gene mutation in the teichoic acids alanylation system of *Lactococcus garvieae* results in diminished proliferation in its natural host., *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.12.004

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1 ***DltA* gene mutation in the teichoic acids alanylation system of**  
2 ***Lactococcus garvieae* results in diminished proliferation in its natural**  
3 **host.**

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## Abstract

The *dltABCD* cluster is involved in the D-alanylation of teichoic acids in gram positive bacteria. In order to determine the role of this alanylation in the physiology and virulence of *Lactococcus garvieae*, a previously isolated *dltA*ΔTn917 signature tagged mutagenesis (STM) clone was analyzed. RT-PCR results revealed that *dltABCD* genes form an operon. No major differences could be established between the parental and mutant strains with respect to growth rate, autolytic properties, and susceptibility to acid conditions, lysozyme treatment, anionic detergents, or oxidant agents. However, the *dltA* mutant was more susceptible to nisin than the parental strain, with minimum inhibitory concentration (MIC) values of 8 µg/ml and 16 µg/ml, respectively. Less proliferation of the mutant was observed in *in vivo* competence index experiments (CI= 0.08). Furthermore, the mutant strain had a 50% lethal dose (LD<sub>50</sub>) three-fold that of the parental strain. These results, together with the fact that the *dltA*ΔTn917 mutant was isolated as a STM clone, reveal that the *dltA* locus of *L. garvieae* is required for full growth and pathogenesis on rainbow trout.

Keywords: *Lactococcus garvieae*; *dltA*; host-limited replication

## 1 1. Introduction

2

3 *L. garvieae* is the etiological agent of lactococcosis that causes hyperacute,  
4 haemorrhagic septicaemia in many fish species the world over with special  
5 incidence in rainbow trout (*Onchorhynchus mykiss*) and yellowtail (*Seriola*  
6 *quinqueradiata*) aquaculture (Vendrell et al., 2006). Moreover, this  
7 microorganism is ubiquitous, as it has also been isolated in buffaloes suffering  
8 from mastitis (Teixeira et al., 1996) and even in humans, associated with  
9 bacterial endocarditis and with different tissue infections (Fihman et al., 2006; Li  
10 et al., 2008). This suggests that this bacterium might be considered an  
11 emerging pathogen and potential zoonotic agent.

12

13 Despite the importance of this pathogen, little is known about the molecular  
14 basis of its mechanisms of virulence. According to several studies, the presence  
15 or absence of a capsule is a major determinant of virulence (Barnes et al.,  
16 2002) and putative siderophores have been reported in *L. garvieae* (Schmidtke  
17 and Carson, 2003).

18

19 *dlt* operon gene products reportedly participate in D-alanylation of teichoic  
20 acids in many gram positive bacteria (Neuhaus and Baddiley, 2003) and  
21 disturbances of this activity have been shown to produce a wide variety of  
22 phenotypic changes. Additionally, a correlation has been established between  
23 D-alanylation of teichoic acids and virulence in *Listeria monocytogenes*  
24 (Abachin et al., 2002), *Streptococcus agalactiae* (Poyart et al., 2003), and  
25 *Staphylococcus aureus* (Collins et al., 2002).

26

27 Here we report the identification and sequence of the *dlt* loci in *L. garvieae*. RT-  
28 PCR analyses have indicated that said genes form a *dltABCD* operon.  
29 Characterization of a *dltAΔTn917* mutant showed that the bacterium presents  
30 an alteration of the cell surface charge and increased susceptibility to nisin in  
31 comparison to the parental strain. Furthermore, the growth capacity of the  
32 bacterium was severely impaired in rainbow trout, its natural host.

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## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The strains used in this study were *L. garvieae* UNIUD074 (obtained from Dr L. Gusmani, University of Udine, Italy) and mutant XVIII (Menéndez et al., 2007). *L. garvieae* strains were routinely cultured at 20 C° in brain heart infusion (BHI) broth (Difco). Erythromycin, 1 µg/ml, was added to the medium when growing mutant XVIII. BHI plates contained 2% agar.

### 2.2. DNA manipulation

Chromosomal DNA extraction was conducted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich Co.) following the manufacturer's instructions. Routine DNA manipulation was performed as described by Sambrook & Russell (2001). Phage T4 DNA ligase, calf intestinal alkaline phosphatase, and restriction enzymes were purchased from Amersham, and oligonucleotides were from Sigma-Aldrich Co.

### 2.3. Identification of genomic regions flanking Tn917 insertion in mutant XVIII

Tn917 insertion in mutant XVIII occurred in a gene encoding a protein homologous to DltA (Menéndez et al., 2007). The inverse PCR method (IPCR) was used to amplify Tn917-DNA flanking regions. *L. garvieae* UNIUD074 genomic DNA was digested with *Pst*I at 37°C for 5 hours. The digested DNA was then circularized at 16°C overnight and the reaction mix was used as the template for IPCR under the following conditions: 10 min of denaturation at 94°C, 35 1-min cycles at 94°C, 1 min at 60°C, and 10 min at 72°C, followed by 10 min of final extension at 72°C. Primers dltA1: 5' GGTCCACCCGTTGTCAAGC 3' and dltA2: 5' GCTATCACAAGTATTCA 3', designed from the known *dltA* sequence of *L. garvieae* (Menéndez et al., 2007), were used in the amplification process. The PCR product was purified with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and the

1 sequence was obtained in an ABI Prism 310A automated DNA sequencer from  
2 Perkin-Elmer, according to the manufacturer's instructions, at the facilities' of  
3 the Universidad de Oviedo. Sequence analysis was performed using the  
4 BLASTX computer program (<http://blast.ncbi.nlm.nih.gov/>).

5

#### 6 *2.4. RNA isolation and RT-PCRs*

7

8 Total RNA was obtained from 2 ml overnight cultures of parental and mutant  
9 strains grown in BHI. RNA was isolated using an RNeasy Mini Kit (QIAGEN,  
10 Valencia, CA.) and treated with DNase I-RNase-free (Ambion, Inc.) to eliminate  
11 traces of DNA. Reverse transcriptions (RT-PCRs) were performed using  
12 Superscript One-Step with Platinum Taq (Invitrogen Life Technologies,  
13 Carlsbad, CA.). Seventy nanograms of RNA were used in each reaction.  
14 Control PCRs using Platinum Taq DNA polymerase (Invitrogen Life  
15 Technologies, Carlsbad, CA.) were carried out to determine whether RNA was  
16 free of contaminant DNA and negative controls were included in each run by  
17 omitting the reverse transcription step. The primers used for RT-PCR reactions  
18 are listed in Table 1.

19

#### 20 *2.5. Alcian Blue binding assay*

21

22 The binding capacity of the cationic colorant Alcian blue to both strains was  
23 determined using the method described by Chan et al. (2007) with slight  
24 modifications. Briefly put, cells grown in BHI and adjusted to an optical density  
25 (OD) at 600 nm of 0.4, were harvested and washed once by centrifugation  
26 (10,000 x *g* for 5 min) with 20 mM morpholinepropanesulfonic acid (MOPS)  
27 buffer (pH 7). The cells were resuspended in MOPS buffer to a final OD at 600  
28 nm of 0.4, and the cationic dye Alcian blue 8GX (Sigma Aldrich Co.) was added  
29 to a final concentration of 250 µg/ml. Samples were rotated at room  
30 temperature. After 10 min, the mixtures were centrifuged to pellet the bacteria  
31 and the unbound Alcian blue present in the supernatants was measured at  
32 OD<sub>615</sub> nm. In parallel, tubes containing the same amount of Alcian blue in  
33 MOPS buffer but without bacteria were treated in identical manner as controls.  
34 The amount of Alcian blue bound to the bacteria was calculated as (OD<sub>615</sub> of

1 control supernatant –  $OD_{615}$  of sample supernatant) /  $OD_{615}$  of control  
2 supernatant x 100. Each experiment was carried out three times.

3

#### 4 2.6. Antimicrobial susceptibility test

5

6 Antimicrobial susceptibility tests were performed according to the broth  
7 microdilution methods described by the Clinical and Laboratory Standards  
8 Institute (CLSI, 2006). Three antimicrobial agents were used: nisin (0.0156–16  
9  $\mu\text{g/ml}$ ), vancomycin (0.0156–16  $\mu\text{g/ml}$ ), and sodium colistimethate (16-4,096  
10  $\mu\text{g/ml}$ ). Antibacterial agents were serially diluted twice in cation-adjusted  
11 Mueller-Hinton broth (CAMHB) in U-bottom 96-well microtiter plates. For  
12 inoculation, 0.5 McFarland suspensions of the isolates were diluted 10-fold with  
13 CAMHB. Diluted inocula (5  $\mu\text{l}$ ) were added to each microtiter well and incubated  
14 at 28°C for 24 h. Each panel contained a negative control well without  
15 antibiotics or inoculum. The lowest concentration of antibiotic that visibly  
16 inhibited bacterial growth was regarded as the MIC (CLSI, 2006). Currently, no  
17 criteria have been established for interpreting MIC test results in aquatic  
18 pathogens (resistant, intermediate, or susceptible breakpoints). Each  
19 experiment was carried out three times.

20

#### 21 2.7. Competition experiments and $LD_{50}$ determination

22

23 For each *in vivo* experiment, rainbow trout (*Oncorhynchus mykiss* weighing  
24 between 10 and 15 g) were obtained from a commercial fish farm and the  
25 animals were maintained in 60-litre plastic tanks supplied with well-aerated  
26 dechlorinated water at 18°C.

27 For *in vivo* competition assays, parental strain UNIUD074 and mutant *XVIII*  
28 were grown separately in BHI at 20°C and adjusted to an OD at 600 nm of 0.5  
29 ( $10^8$  CFU/ml). Bacteria were harvested and washed in phosphate-buffered  
30 saline (PBS) and mutant *XVIII* was mixed with the parental strain. Dilutions of  
31 the resultant suspension were plated onto BHI (to measure total CFU) and BHI  
32 with erythromycin (to determine mutant CFU). From this, the exact mutant to  
33 parental input ratio was calculated. A sample of  $10^4$  CFU of the mixed  
34 suspension in 0.1 ml was used to infect ten rainbow trout by intraperitoneal

1 injection. After 72 h, three animals were sacrificed and dissected, and the liver  
2 and spleen were homogenized in BHI with a stomacher. Homogenates were  
3 plated onto selective media to determine the mutant to parental output ratio. CI  
4 is defined as the output ratio (mutant/parental) divided by the input ratio  
5 (mutant/parental). Each experiment was carried out twice.

6  
7 For 50% lethal dose (LD<sub>50</sub>) determinations, groups of 10 fish were challenged  
8 by intraperitoneal injection of 0.1-ml serial tenfold dilutions of exponential-phase  
9 cultures of the strains *L. garvieae* UNIUD074 and mutant *XVIII* corresponding to  
10 a range of 10<sup>2</sup> to 10<sup>7</sup> CFU. The microorganisms were previously washed and  
11 resuspended in PBS. A group of ten fish injected with 0.1 ml of PBS was used  
12 as controls. Mortality of the fish was recorded over a period of 7 days post  
13 injection and some dead specimens were removed and dissected for routine  
14 bacteriological examination. Samples of the spleens and livers were plated onto  
15 BHI and BHI with erythromycin; PCR analysis confirmed that *L. garvieae*  
16 colonies were present. Each experiment was carried out twice and LD<sub>50</sub> were  
17 calculated three days post injection using Reed and Muench's statistical  
18 approach (1938). All of the animal experiments were conducted in compliance  
19 with European legislation governing animal welfare and were authorized and  
20 supervised by the Animal Experimentation Ethics Committee from the Oviedo  
21 University.

#### 22 23 *2.8. Nucleotide sequence accession number*

24  
25 The sequence accession number for the *dlt* genes in the GenBank database is  
26 FJ973474.

### 3. Results and discussion

The application of STM to *L. garvieae* UNIUD074 led to the identification of 29 mutants that could not be recovered from rainbow trout following infection (Menéndez et al., 2007). Among them, mutant XVIII, which showed a single Tn917 insertion in a *dltA* gene homologue, underwent further analysis. The IPCR method was used to amplify the Tn917-DNA flanking regions. Sequence analysis of the amplicon obtained led to the identification of a 4,700 bp DNA segment containing five open reading frames (ORF), all in the same direction. The deduced amino acid sequence of the first ORF revealed 39% identity to the hypothetical protein L170548 of *Lactococcus lactis* subsp. *lactis* Il1403 (accession number NP267886). This protein has an alpha/beta hydrolase domain whose function has yet to be characterized and is encoded by the *yriC* gene. The products of the transduction of the other four ORFs disclosed significant identities to the proteins encoded by the *dlt* region, which has been seen to be involved in the D-alanylation of teichoic acids in many gram positive bacteria. These proteins are: DltA (with 62% identity to the DltA protein of *Lactococcus lactis* subsp. *cremoris* MG1363), DltB, DltC, and DltD (revealing an identity of 69%, 80%, and 49% to the DltB, DltC and DltD proteins of *L. lactis* subsp. *lactis* Il1403, respectively). *DltA* gene product is a protein containing 501 amino acids (Accession number FJ973474); the Tn917 transposon insertion into the gene occurred in the coding sequence at amino acid position 363. This means that the Tn917 insertion site precedes two of the three regions proposed by Heaton and Neuhaus (1992) as being essential to *dltA* gene function. In order to examine the possibility of there being more than one copy of the *dlt* genes, Southern Blot analysis of *Bam*HI digested genomic DNA from *L. garvieae* UNIUD074 was conducted using an internal labelled PCR *dltA* generated fragment as a probe. A single hybridization band was observed suggesting the presence of a single locus for the *dlt* operon (data not shown). The *dltABCD* cluster in *L. garvieae* is flanked by two transcriptional terminators (Fig. 1a). Analysis of *dltABCD* genetic organization showed that *dltB* overlaps the stop codon of *dltA* by 1 bp, whereas *dltB* and *dltC* are separated by 24 bp and *dltD* overlaps the stop codon of *dltC* by 14 bp. Therefore, a translation coupling mechanism previously described in other *dlt* operons may be also

1 present in *L. garvieae* to provide coordinated synthesis of Dlt proteins.  
2 However, it is worth noting that presumed ribosome binding sites (RBS)  
3 preceded the *dltA* (GGAG), *dltB* (GGAGG), *dltC* (GAAG), and *dltD* (GGA) start  
4 codons (Fig. 1a). Hence, the *dltABCD* gene translation in *L. garvieae* might also  
5 be specific for each gene and independent from the preceding genes. This  
6 putative RBS configuration is also present in the *dlt* operon of *L. lactis*  
7 (accession number AE008562) and *S. agalactiae* (accession number  
8 AJ291784). Further experiments are needed to determine the translational  
9 process of the *dlt* operon.

10  
11 The small size of the intergenic spaces, together with the presence of putative  
12 transcriptional terminators flanking the *dltABCD* gene cluster of *L. garvieae*,  
13 suggested that it might be transcribed as a single unit as occurs in other gram  
14 positive bacteria. This was confirmed by RT-PCR analyses using the primers  
15 listed in Table 1. Fragments corresponding to the overlapping regions of the  
16 *dltABCD* genes were amplified when RNA from the parental strain *L. garvieae*  
17 UNIUD074 was used (Fig. 1b), revealing that the *dlt* loci form an operon. When  
18 RNA from the *dltA*ΔTn917 strain was used in RT-PCR analysis, the *dltA* gene  
19 was not amplified (Fig. 1c). Nevertheless, amplifications were obtained  
20 corresponding to the overlapping region *dltA-dltB* and to an internal region of  
21 the *dltB* locus (Fig. 1c). This indicates that the *dltA* mutation resulting from the  
22 Tn917 insertion does not have a polar effect. It could be due to the fact that the  
23 transposon Tn917 has some kind of promoter activity. Thus, Dobinsky et al.  
24 (2002) observed that transposon Tn917 allowed transcriptional read-through  
25 into downstream located genes when Tn917 inserted in the same transcriptional  
26 direction as those genes, as occurred in the *dltA*ΔTn917 strain. However, the  
27 translational coupling may compromise the expression of *dltA* downstream  
28 genes.

29  
30 In several previous studies, defects in the D-alanylation of teichoic acids were  
31 hypothesized to result in a more negative cell surface charge, since the  
32 positively charged amino groups of D-alanyl esters partially antagonize the  
33 negative charges of the backbone phosphate groups (Neuhaus and Baddiley,  
34 2003). To examine this, the binding capacity of cationic dye Alcian blue to both

1 strains was determined following the method previously described by Chan et  
2 al. (2007). The results from the Alcian blue binding assay revealed that the  
3 mutant has an increased negative surface charge. Therefore, the *dltA*ΔTn917  
4 strain bound 89.9% ± 1.15 of the Alcian blue versus 80.48% ± 2.44 in the case  
5 of the parental strain. These values differ statistically ( $P < 0.05$ ) and are similar to  
6 the ones found in the Alcian blue binding assay of *Streptococcus gordonii dltA*  
7 mutants (Chan et al., 2007). These results are also consistent with those  
8 obtained in the cytochrome c binding assay of *Bacillus subtilis dlt* mutants  
9 (Wecke et al., 1997), which reflected an increase in anionic binding sites on the  
10 bacterial surface, indicating that the cell's surface charge had been modified  
11 considerably.

12  
13 Teichoic acids are expected to bear a higher negative charge if defects in D-  
14 alanyl substitutions occur; consequently, *dlt* mutants in several gram positive  
15 bacterial species exhibit enhanced sensitivity to antimicrobial cationic peptides  
16 (Peschel et al., 1999; Kristian et al., 2005; Kovacs et al., 2006; Perea Velez et  
17 al., 2007). Thus, the parental and *dltA* mutant strains were tested for  
18 susceptibility to the antibiotics nisin, vancomycin, and sodium colistimethate.  
19 Thanks to their increased negative cell surface charge, *dlt* mutants bind  
20 positively charged compounds such as the cationic antimicrobial nisin more  
21 avidly, resulting in increased sensitivity. Accordingly, the MIC determination of  
22 nisin showed that the *dltA* mutant is more sensitive to the antibiotic than the  
23 parental strain, with values of 8 µg/ml and 16 µg/ml, respectively. Sensitivity to  
24 nisin in gram positive bacteria depends on different mechanisms. Thus, in *S.*  
25 *aureus*, the absence of D-alanylation leads to increased sensitivity to nisin  
26 (Peschel et al., 1999), albeit not in *L. monocytogenes* (Davies et al., 1996) or in  
27 *Streptococcus bovis* (Mantovani and Russell, 2001). On the contrary, no  
28 differences were observed in MIC values between parental and mutant strains  
29 for vancomycin (MIC 0.5 µg/ml) and sodium colistimethate (MIC 4,096 µg/ml). It  
30 should be noted that gram positive bacteria are less susceptible than gram  
31 negative bacteria to sodium colistimethate and other polymyxin antibiotics due  
32 to the protective effect of their cell wall. On the other hand, other cell surface  
33 modifications may also contribute to resistance to antimicrobial peptides.  
34 Similarly, deacetylation of the hexosamine residues of the peptidoglycan by

1 *PgdA* introduces positive charges into murein in *Streptococcus pneumoniae*  
2 (Vollmer and Tomasz, 2000) and the modification of anionic membrane lipids  
3 with L-lysine by *MprF* decreases the negative charge of the membrane surface  
4 in *S. aureus* (Peschel et al., 2001). Thus, a group of several mechanisms may  
5 be responsible for conferring overall resistance to antimicrobial peptides and the  
6 role D-alanylation of teichoic acids plays in resistance may vary considerably  
7 from one bacterial species to another ( Peschel et al., 1999; Abachin et al.,  
8 2002; Poyart et al., 2003).

9  
10 Comparison of the growth curves of the parental and *dltAΔTn917* strains  
11 showed no differences (data not shown). No differences were detected in their  
12 autolytic properties either as per the induced autolysis assay described by  
13 Perea Velez et al. (2007) (data not shown). Likewise, both strains behaved  
14 identically when grown on BHI media under acid conditions and presented  
15 equal MIC values to lysozyme, SDS, and H<sub>2</sub>O<sub>2</sub> (data not shown). These results  
16 contrast those of previous reports that reported differences in growth rates  
17 (Clemans et al., 1999), induced autolysis (Walter et al., 2007), and sensitivity to  
18 acid conditions (Walter et al., 2007), lysozyme (Giaouris et al., 2008), anionic  
19 detergents (Perea Vélez et al., 2007), or oxidant agents between parental  
20 strains and *dlt* mutants of gram positive bacteria.

21  
22 Transmission electron microscopy was performed with cells collected in  
23 exponential growth and late stationary phases. No dramatic changes in cell  
24 morphology were seen in the *dltAΔTn917* mutant strain populations , as occurs  
25 in *Lactobacillus plantarum* (Palumbo et al., 2006), although some cells  
26 presented irregular shapes which were not observed in the parental strain  
27 populations (Fig. 2). This variety of physiological differences between *dlt*  
28 mutants and parental strains is observed in other gram positive bacteria  
29 (Kovacs et al., 2006; Fittipaldi et al., 2008), given that, while it is apparent that  
30 the D-alanyl esters of teichoic acids play an important role in the physiology of  
31 the individual species, there is no single phenotype common to all the species  
32 examined (Neuhaus and Baddiley, 2003).

1 Despite the absence of any salient physiological changes related to the  
2 inactivation of the *dltA* gene, experiments using rainbow trout made it possible  
3 to evaluate the specific contribution of D-alanylation of teichoic acids to *L.*  
4 *garvieae* pathogenesis. CI constitutes an alternative measuring tool to evaluate  
5 the degree of growth due to a given mutation in mutant and parental strain  
6 coinfections. A value of  $0.082 \pm 0.1$  was obtained in the *in vivo* competition  
7 experiments. This result clearly establishes that the mutant strain has  
8 significantly greater difficulty growing inside the host than the parental strain.

9  
10 The mean LD<sub>50</sub> values obtained for the parental and mutant strains were  $5.2 \times$   
11  $10^3$  and  $4.2 \times 10^6$  CFU per fish, respectively. Furthermore, following a seven-  
12 day post injection period, mean survival rates indicated that fish infected with  
13 the mutant strain survived longer than those infected with the parental strain  
14 (data not shown). This demonstrates that the three-fold LD<sub>50</sub> value of the  
15 *dltA*ΔTn917 mutant strain versus the parental strain, is not a consequence of  
16 attenuated virulence, but rather is due to the bacterium's limited capacity for  
17 replication within the host. It is tempting to speculate that this might be a  
18 consequence of greater sensitivity of the mutant strain to the peptide antibiotics  
19 called cathelicidins, which are produced by rainbow trout and are involved in the  
20 innate immune response of the fish (Chang et al., 2006). Because they are not  
21 commercially available as yet, they could not be assayed in MIC experiments.

22  
23 *Dlt* mutants of different gram positive pathogens have been described as highly  
24 susceptible to death by neutrophils and/or by macrophages (Abachin et al.,  
25 2002; Poyart et al., 2003; Kristian et al., 2005; Weidenmaier et al., 2005; Chan  
26 et al., 2007) and present reduced biofilm formation (Gross et al., 2001) or  
27 adherence (Abachin et al., 2002; Kristian et al., 2005). Furthermore, D-  
28 alanylation of teichoic acids has been seen to contribute to virulence in several  
29 gram positive bacteria in surrogate models of infection (Abachin et al., 2002;  
30 Collins et al., 2002; Poyart et al., 2003; Weidenmaier et al., 2005), but only one  
31 *dltA* mutant of *Streptococcus suis* was tested in its natural host (Fittipaldi et al.,  
32 2008). Therefore, D-alanylation of teichoic acids may be involved in virulence in  
33 bacteria by means of a combined action, interfering with the host's innate  
34 immune clearance mechanisms and facilitating bacterial penetration through the

1 natural host's barriers. In this study we report what is, to our knowledge, the first  
2 evidence of the importance of D-alanylation of teichoic acids in the  
3 pathogenesis of *L. garvieae*.

4  
5  
6 **Aknowledgements.** This work was supported by a grant from the MICINN of  
7 Spain (AGL2006-07562). J. Méndez was the recipient of a grant from the  
8 University of Oviedo. P. Reimundo, D. Pérez-Pascual and R. Navais were  
9 recipients of predoctoral fellowships from the Spanish Ministerio de Ciencia e  
10 Innovación. We thank Dr. Laura Gusmani from the University of Udine, Italy, for  
11 donating the *L. garvieae* UNIUD074 strain, C. Álvarez for his help in performing  
12 the transmission electron microscopy work and L. Erice for correcting the  
13 manuscript.

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3**Table 1.** Primers used for RT-PCR reactions

Primer	Sequence	Gene
1F	5' GTAACGGGATTGATAGCGGTC 3'	<i>dltA</i>
1R	5' CGGAAACGATCAATTGGCCC 3'	<i>dltB</i>
2F	5' GGTGTTTATCATGCCTGTCTG 3'	<i>dltB</i>
2R	5' TGTTTCGAGTTCAGAAGAGCCT 3'	<i>dltD</i>
3F	5' AGCCAAGAGGAGACAACG 3'	<i>dltA</i>
3R	5' CAACTCGATTCTAAAACC 3'	<i>dltA</i>
4F	5' GCTCTACGCGCACTATCGAA 3'	<i>dltB</i>
4R	5' CAGACAGGCATGATAAACACC 3'	<i>dltB</i>

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**Figure 1.**

**RT-PCR analysis showing transcriptional organization of the genomic *dltABCD* regions flanking Tn917 insertion.**

(a) The orientation of the genes and the location of the primers used for RT-PCR amplifications are shown. P, putative promoter region contains a -10 “TTTAAT” and a -35 “AAGACA” sequences; RBS, putative ribosome binding site; stem-loop structure, putative transcriptional terminator. The insertion site of transposon Tn917 in *dltA* locus of mutant *XVIII* is also indicated.

(b) RT-PCR of *L. garvieae* parental strain using primers 1F and 1R (lane 3), and 2F and 2R (lane 4). (c) RT-PCR of *L. garvieae* parental (lane 2) and mutant (lane 5) strains using primers 3F and 3R to confirm the Tn917 interruption of *dltA* locus in mutant *XVIII*. RT-PCR of mutant strain using primers 1F and 1R (lane 6) and 4F and 4R (lane 7) to analyze whether the *dltA* mutation has a polar effect or not. Lanes 1 (b) and 3 (c) correspond to a 100 bp molecular weight marker. Lanes 2 (b), 1 (c), and 4 (c), negative controls included in each run by omitting the reverse transcription step to determine whether RNA was free of contaminant DNA.

1 **Figure 2**

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4 **Cell morphology of the parental (WT) and *dltA*ΔTn917 strains.**

5

6 Transmission electron microscopy was performed with cells collected in  
7 exponential growth and late stationary phases. Cell samples were washed once  
8 with Tris-HCl buffer 0.1M, pH 7.5 and resuspended in the same buffer. The  
9 samples were observed on a MET JEOL-2000 EX-II transmission electron  
10 microscope after 4% uranyl acetate staining. Some cells in the mutant  
11 populations presented slightly irregular shapes.

12 Bars: 0.5 μm WT and *dltA*ΔTn917.

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Figure 1

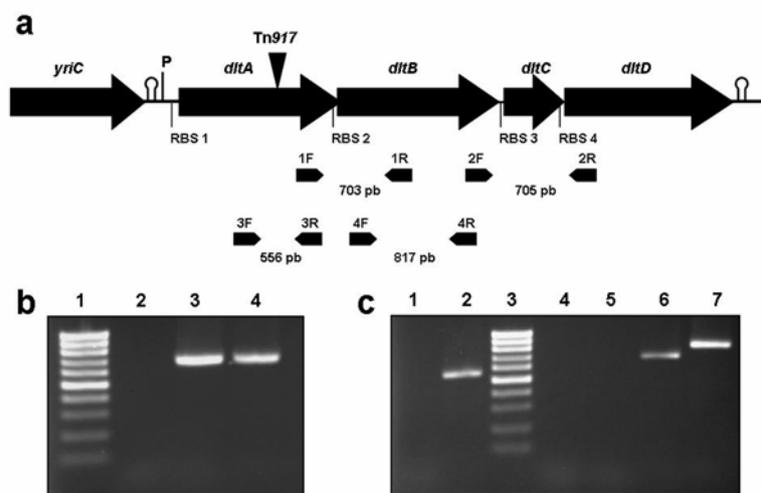
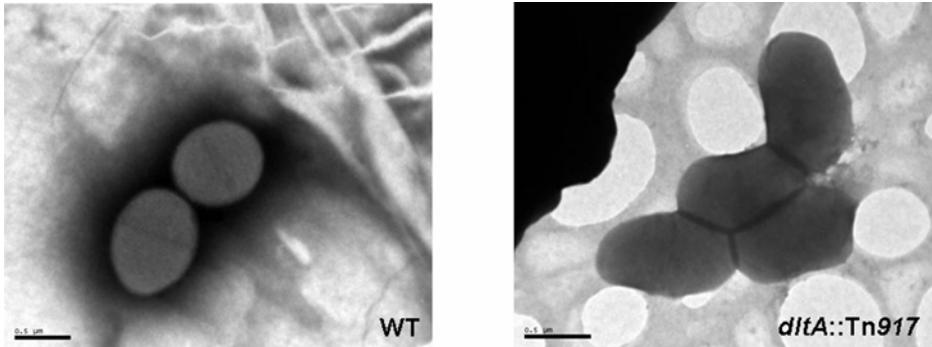


Figure 2



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