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Colonization capacity and serum bactericidal activity of *Haemophilus parasuis thy* mutants

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Summary. The bacterial *thyA* gene encodes the enzyme thymidylate synthase, which is essential for dTMP synthesis and, consequently, for DNA replication. In this work, a *Haemophilus parasuis thyA* mutant was constructed in order to analyze its colonization characteristics and its capacity to generate serum bactericidal activity in infected guinea pigs. The data showed that colonization by the *H. parasuis thyA* mutant was much less than that of the wild-type strain. Nevertheless, the mutant generated a strong immunogenic response in the host, as detected by measuring serum bactericidal activity. [**Int Microbiol** 2006; 9(4):297-301]

Key words: *Haemophilus parasuis* · colonization capacity · bactericidal activity · *thy* mutants · vaccine strains · immunogenic response

Introduction

Haemophilus parasuis is a gram-negative bacterium belonging to the *Pasteurellaceae* family. This organism is an important respiratory-tract pathogen in swine and the etiological agent of porcine poliserositis and arthritis syndrome, known as Glässer's disease [11]. *H. parasuis* causes high mortality and morbidity in pig farms, leading to significant economic losses. Although several approaches have been tested [19,25], the development of an effective general vaccination against *H. parasuis* has yet to be achieved.

Thymine is an absolute requirement for chromosome bacterial replication; thus, its biosynthesis is essential for bacterial cell survival and growth. Bacteria synthesize deoxythymidylate monophosphate nucleotide (dTMP) via the methylation of deoxyuridylate (dUMP). The reaction is catalyzed by thymidylate synthase, which is encoded by the *thyA* gene [3,23]. Bacterial *thyA* mutants cannot replicate their DNA in the absence of exogenous precursors, such as thymine or thymidine, in the growth medium [18] and they eventually die. In fact, inactivation of the *thyA* gene has been proposed as a strategy for the biological containment of bacteria in the environment [15]. Nevertheless, analyses of the protection and colonization abilities of *thyA* mutants from different bacterial pathogens have yielded contradictory results. For example, *Thy* mutants of *Legionella pneumophila*, which inhabit phagosomes [2], lose their infectivity in monocyte cultures [17]. By contrast, the virulence of *Salmonella enterica* Typhimurium *thy* mutants is recovered in the presence of the appropriate thymine concentration in the host [13]. It has been determined that *thyA* mutants of *Shigella*

flexneri are much less virulent than wild-type [1,5,20], whereas in the baby-mouse cholera model *Vibrio cholerae thy* mutants are able to colonize the animals and are immunogenic in rabbits as well [26]. Taken together, these findings show that the pathogenicity of bacterial *thy* mutants depends on the bacterial species. In this study, the colonization capacity of a *H. parasuis thyA* mutant and its ability to induce an immunogenic response in a guinea pig model of lung infection were analyzed.

Materials and methods

Bacterial strains and growth conditions. Bacteria used in this study are listed in Table 1. *Escherichia coli* strains were grown in LB medium [24], and *H. parasuis* on chocolate-blood agar plates (Biomérieux) and in tryptone-yeast extract (TYE) broth [21]. When necessary, *H. parasuis* strains were grown in the presence of kanamycin (50 μg/ml), gentamycin (20 μg/ml), or trimethoprim (100 μg/ml).

Genetic methods. The *H. parasuis thyA* mutant was obtained by natural transformation as previously described [4]. Briefly, the *H. parasuis thyA* gene was isolated by complementation of an *E. coli thyA* mutant using a genomic library constructed with the broad-host-range vector pUA520. Among several plasmids of the library that were able to complement the *E. coli thyA* gene, one of them was kept and sequenced to confirm the presence of the *H. parasuis thyA* gene (GenBank accession number AY262733). A 0.8-kb fragment encoding the kanamycin resistance gene from the Tn5 transposon was cloned into the *H. parasuis thy* gene in internal *Eco*RI sites previously generated by PCR overlap extension. The *thy*::Km fragment was then cloned into the suicidal and narrow-host-range plasmid pUA658, which was transformed into *H. parasuis* cells (Fig. 1A). The resulting Km^R and Gm^S clones were analyzed by PCR to confirm that the wild-type *thy* gene had been replaced by the mutant copy (Fig. 1B), using primers Thyup 3′-TACTCCTTATCTCTCTGTAC-5′ and Thydw 3′- AATAAAGTCTGTAAGATAGC-5′. In concordance with the fact that mutations in the thymidylate synthase gene (*thyA*) confer resistance to trimethoprim in *E. coli* [16], the *H. parasuis thy* mutant, but not the wild-type, was able to grow on chocolate-blood agar plates supplemented with trimethoprim (Fig. 1C).

Animal experiments. Guinea pigs were used as the animal model. The animals were kept at room temperature in individual enclosures, acclimated for 6 days prior to inoculation, and received water and food *ad libitum*. The inoculations were administered intratracheally [22] and consisted of 0.2 ml of bacterial suspension in TYE broth, equivalent to 10^{10} cfu/ml. The animals were killed at 1, 4, 7, and 11 days after inoculation by intraperitoneal injection of pentobarbital. Before the first inoculation, one animal was killed and used as a control. Blood samples were collected from the heart of the guinea pig immediately before the animals were killed. Sera were incubated at 37ºC, centrifuged, aliquoted, and stored at –20ºC until use. To recover *H. parasuis* from infected animals, samples were taken aseptically from guinea-pig lungs. Each lung sample was divided into two equal-sized parts. One part was stored at –80ºC for DNA extraction using the DNeasy Tissue Kit (Qiagen) followed by PCR amplification with primers described earlier [10]. The other part was homogenized and plated onto chocolate-blood agar plates, which were incubated at 37ºC in an atmosphere containing 5% carbon dioxide.

Protein and immunoblot studies. To detect antibodies against *H. parasuis*, outer-membrane proteins from the wild-type strain were extracted as previously reported [8], with minor modifications. Protein concentration was measured as described before [14] and protein profiles were examined by 12% SDS-PAGE [12]. The antigenicity of sera was determined by Western blot analysis as previously described [8], with slight modifications. Briefly, the SDS-PAGE gels of outer-membrane proteins extracted from the *H. parasuis* wild-type were transferred to Immobilon-P membranes (Millipore) using a Hoefer miniVe (Amersham Pharmacia Biotech) TransBlot Cell. Membranes were air-dried for 20 min and blocked for 2 h in blocking solution (200 ml PBS buffer, 2 g blocking reagent, and 0.2 ml Tween 20). The transferred proteins were immunostained overnight with a specific serum at a dilution of 1/100 in blocking solution. Afterwards, membranes were washed three times with PBS and incubated in a 1/30,000 dilution in blocking solution of anti-guinea pig IgG (Sigma) for 1 h. Membranes were again washed three times with PBS, and reactive polypeptides were visualized in alkaline phosphate buffer (100 mM NaCl, 50 mM Tris/HCl, 5 mM $MnCl_2$) containing 4-nitro blue tetrazolium chloride and X-phosphate-

Table 1. Bacterial strains and plasmids used in this work

	Relevant features	Source or reference
Bacterial strains		
Escherichia coli		
DH5α	*supE4* Δ*lacU169* (Ø80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[24]
KL742	λ⁻ *thyA*748::Tn10 *rph-1 deo-77*	CGSC(Coli Genetic Stock Center)
Haemophilus parasuis		
HP100	Parenteral strain, serotype 5, isolated from a naturally infected pig	I. Badiola (IRTA,Spain)
HP102	HP100 *thy* Km^R	This laboratory
Plasmids		
pBBR1MCS	A broad-host-range cloning vector Cm^R Mob^+	BioTechniques
pGEM-T	PCR cloning vector Ap^R	Promega
pGP704	Suicide and narrow-broad-host vector Ap^R	[9]
pRK2013	Mob^+ Tra^+ Km^R	[6]
pUA520	pBBR1MCS Km^R	This laboratory
pUA658	pGP704 Gm^R	This laboratory
pUA1059	pUA658 carrying a 4-kb fragment containing the *H. parasuis thyA* gene interrupted with Km cassette Km^R	This work

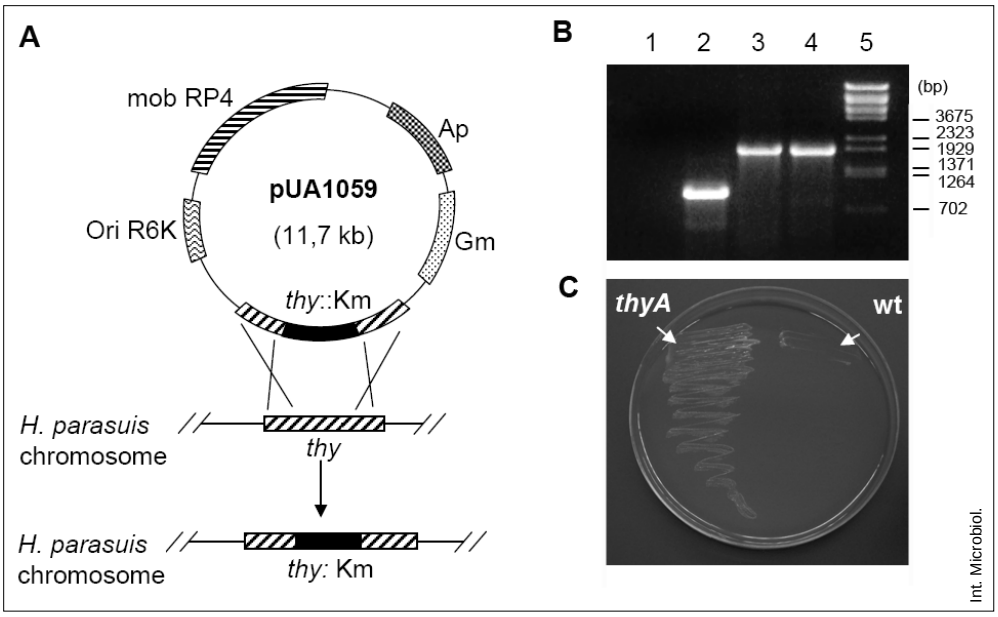

Fig. 1. Construction of the *Haemophilus parasuis thyA* strain. (**A**) Mutant construction. The *thy*::km fragment was cloned into suicidal vector pUA658, yielding pUA1059. The construct was introduced into *H. parasuis* wild-type by natural transformation. (**B**) PCR analysis of chromosomal DNA from the *H. parasuis thyA* mutant using Thyup and Thydw oligonucleotides. The results of PCR reactions using chromosomal DNA from *H. parasuis thyA* (lane 4), pUA1059 vector (lane 3), and chromosomal DNA of the *H. parasuis* wild-type strain (lane 2), as well as the negative control (lane 1). *Bst*EII digested-λ DNA was used as the molecular size marker (lane 5). (**C**) Phenotypic test of the presence of the *thyA* mutation. The Thy mutant, but not the wild-type strain, was able to grow on plates supplemented with trimethoprim.

5-bromo-4-chloro-3-indolyl phosphate (BCIP, 4-toluidine salt), as recommended by the supplier (Roche Diagnostics).

Bactericidal assays. The method used was basically as already described [7], with slight modifications. Briefly, 10 µl containing 10^6 cells of the wild-type strain were added to 190 µl of the serum samples, which had been diluted 50% by the addition of PBS, or to the control solution containing PBS alone. All of the samples were incubated at 37ºC with shaking for 30 min. Serial dilutions of each reaction were plated onto chocolate-blood agar, incubated for 24 h, and the resulting colonies were counted.

Results and Discussion

Inactivation of *H. parasuis* colonization ability. *H. parasuis* wild-type or strain *thyA* was detected in the lungs of guinea pigs inoculated with the respective strain, as shown by PCR analysis using oligonucleotides corresponding to an internal sequence of the *H. parasuis* 16S rRNA gene [10]. However, when samples of homogenized infected lungs were plated on chocolate-blood agar plates, only colonies of the *H. parasuis* wild-type strain were recovered (days 4, 7, and 11 after inoculation). These data indicated that bacterial proliferation in the lungs of animals infected with the *thyA* mutant was too low to achieve a cellular concentration high enough to be detected by the plating method. Thus, the colonization capacity of the *H. parasuis thyA* mutant was significantly impaired.

Analysis of serum bactericidal activity induced by *H. parasuis thyA*. To determine the serum dilution factor yielding the greatest decrease in bacterial cell survival, serum from guinea pigs killed 11 days after inoculation with *H. parasuis* wild-type strain was diluted by 10, 25, or 50% and then added to cultures of *H. parasuis* wild-type cells for 30 min. As shown in Fig. 2A, the greatest decrease in cell survival was observed with the 50% serum dilution. Accordingly, this dilution factor was used in subsequent assays.

Sera from guinea pigs killed 1, 4, 7, or 11 days after inoculation with either wild-type *H. parasuis* or the *thyA* mutant were then tested for bactericidal activity against *H. parasuis* wild-type strain. A similar decrease in bacterial survival was noted with sera obtained from wild-type- and *thyA*-infected animals. These results clearly indicate that the immunological response provoked by the two strains in infected animals was the same. In agreement with these data, Western blot analysis demonstrated the presence of antibodies against *H. parasuis* outer-membrane proteins in sera from guinea pigs killed 11 days after infection with either wild-type or *thyA* cells (data not shown).

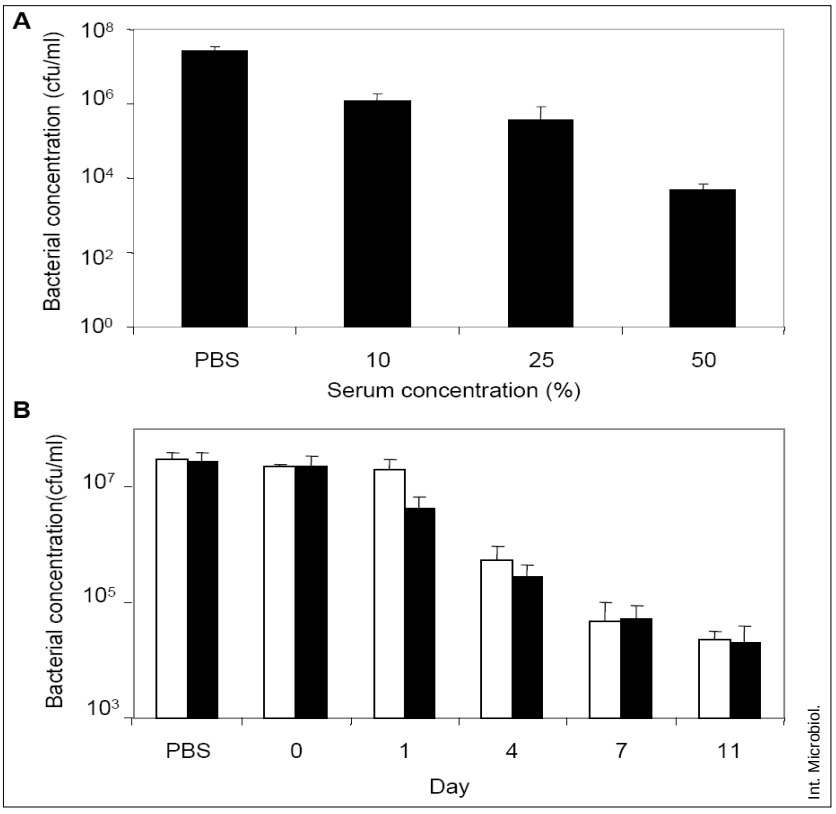

Fig. 2. Bactericidal assays. (**A**) The optimal conditions for demonstrating the bactericidal activity of serum from infected animals were determined by testing 10, 25, or 50% dilutions of serum prepared from guinea pigs killed 11 days after inoculation with the wild-type strain. Maximum bactericidal activity was obtained with the 50% dilution. (**B**) Bactericidal effect of a 50% dilution (in PBS) of sera prepared from guinea pigs infected with either *Haemophilus parasuis* wild-type (black bars) or strain *thyA* (white bars) upon cells of these strains.

The results of this study show that, despite differences in their colonization capacities, *H. parasuis thyA* and wild-type cells are able to induce the same level of serum bactericidal activity in infected guinea pigs. These results provide a basis to evaluate the use of *H. parasuis thyA* mutant as a vaccine strain. Furthermore, our data showed that the permanence time of the *H. parasuis thyA* mutant in the lung of infected animals is sufficient to induce an immunological response by the host.

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Capacidad de colonización y actividad bactericida en suero por mutantes *thy* de *Haemophilus parasuis*

Resumen. El gen bacteriano *thyA* codifica la enzima timidilato sintasa, que es esencial para la síntesis de dTMP y, en consecuencia, para la replicación del DNA. En este trabajo se ha construido un mutante *thyA* de *Haemophilus parasuis* para analizar sus características de colonización, así como la capacidad de generar actividad bactericida en suero en cobayas infectadas. Los datos obtenidos demuestran que la capacidad de colonización del mutante *thyA* de *H. parasuis* es muy reducida con respecto a la cepa tipo salvaje. Sin embargo, el mutante *thyA* de *H. parasuis* origina un elevado nivel de respuesta inmunogénica en el huésped, como se detecta midiendo la actividad bactericida del suero. [**Int Microbiol** 2006; 9(4):297-301]

Palabras clave: *Haemophilus parasuis* · capacidad de colonización · actividad bactericida · mutantes *thy* · cepas vacunales · respuesta inmunogénica

Capacidade de colonização e de geração de atividade bactericida em soro por mutantes *thy* de *Haemophilus parasuis*

Resumo. O gene bacteriano *thyA* codifica a enzima timidilato sintase, que é essencial para a síntese de dTMP e, em conseqüência, para a replicação do DNA. Neste trabalho se construiu um mutante *thyA* de *Haemophilus parasuis* para analisar suas características de colonização, assim como a capacidade de gerar atividade bactericida em soro de cobaias infectadas. Os dados obtidos demonstram que a capacidade de colonização do mutante *thyA* de *H. parasuis* é muito reduzida em relação à cepa tipo selvagem. No entanto, o mutante é capaz de gerar um elevado nível de resposta imunológica no hospedeiro quando se mede atividade bactericida do soro. [**Int Microbiol** 2006; 9(4):297-301]

Palavras chave: *Haemophilus parasuis* · capacidade de colonização · atividade bactericida · mutantes *thy* · cepas vacunales · resposta imunogênica