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To cite this version:

Ambroise Lambert, Jérôme Wong Ng, Mathieu Picardeau. Gene inactivation of a chemotaxis operon in the pathogen Leptospira interrogans. FEMS Microbiology Letters, Wiley-Blackwell, 2015, 362 (3), pp.1-8. 10.1093/femsle/fnu054 . pasteur-02537900

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Submitted on 9 Apr 2020

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Gene inactivation of a chemotaxis operon in the pathogen *Leptospira interrogans*

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ABSTRACT

Chemotaxis may have an important role in the infection process of pathogenic *Leptospira* spp.; however, little is known about the regulation of flagellar-based motility in these atypical bacteria. We generated a library of random transposon mutants of the pathogen *L. interrogans*, which included a mutant with insertion in the first gene of an operon containing the chemotaxis genes *cheA, cheW, cheD, cheB, cheY,* and *mcp*. The disrupted gene encodes a putative histidine kinase (HK). The *HK* mutant was motile and virulent, but swarm plate and capillary assays suggested that chemotaxis was reduced in this mutant. Further analysis of bacterial trajectories by videomicroscopy showed that the ability of this mutant to reverse was significantly impaired in comparison to wild-type strain. Our data therefore shows that this operon is required for full chemotaxis of *Leptospira spp.*

INTRODUCTION

Leptospirosis is a neglected zoonotic disease that shows a worldwide distribution with a high incidence in tropical countries. There are more than one million severe cases per year in the world. The clinical manifestations of leptospirosis are diverse; the spectrum of symptoms ranges from subclinical or mild, anicteric, febrile disease, to multiorgan injury associated with a high probability of mortality if left untreated (Ko*, et al.*, 2009). Leptospirosis is caused by one of the ten pathogenic *Leptospira* spp. described so far. Most mammals can be reservoirs of these bacteria, but wild rodents are usually considered as the main reservoirs for human leptospirosis. Rats are asymptomatic carriers of the bacterium and maintain it as a chronic infection of the renal tubes, excreting bacteria in their urine throughout life (Bharti*, et al.*, 2003). Humans become accidentally infected generally by contact of abraded skin or mucous membranes with water or soil contaminated with the urine of an infected animal (Bharti*, et al.*, 2003). Pathogenic *Leptospira* strains are highly motile bacteria, which can reach target organs in a short period of time (Faine & Vanderhoeden, 1964, Faine*, et al.*, 1999, Ko*, et al.*, 2009). It has also been shown that the velocity of *Leptospira* cells increases with viscosity (Kaiser & Doetsch, 1975, Takabe*, et al.*, 2013). *Leptospira spp.* contains genes encoding chemotaxis proteins, suggesting that motility in *Leptospira* spp. is controlled by a chemotaxis regulatory system that allows the spirochetes to move through viscous media. However, the mechanism of chemotaxis in *Leptospira* spp. and its role in pathogenesis is unknown.

Chemotaxis has been extensively studied in enterobacteria and other model organisms such as *B. subtilis* (Szurmant & Ordal, 2004). Despite some variations, the overall function remains the same: chemotaxis is the movement towards chemoattractant (mostly nutrients) or away from chemorepellents (toxic elements), thus allowing bacteria to avoid a detrimental environment or move to a favorable one. Bacteria developed a complex and robust machinery to achieve this purpose. Methyl-accepting Chemotaxis Proteins (MCPs) are the sensors that bind

to chemical compounds. This signal is then transduced through a phosphorylation pathway in the cytoplasm. CheW is the protein that tethers the sensor kinase CheA to the MCP cytosolic domain. The phosphorylation status of CheA is regulated by the rate of methylation of MCP, which is controlled by CheB (Methylesterase) and CheR (Methylase). CheY binds to the flagellar motor and induces a switch in the sense of rotation of the flagellar motor, and is phosphorylated by the sensor kinase CheA and dephosphorylated by CheZ (Sourjik & Armitage, 2010).

Unlike the spirochete *Borrelia burgdorferi* (Charon*, et al.*, 2012)*,* little is known about the motility and chemotaxis of *Leptospira* spp.*,* which are helix-shaped bacteria with two periplasmic flagella. One flagellum emerges from a basal body at each polar end without overlapping at the cell center. The mechanisms of motility and chemotaxis of *Leptospira* spp. may involve more genes than those of *Borrelia* spp., and are probably more complex, because *Leptospira* spp. have a larger genome (>3.9 Mb) than that of *B. burgdorferi* (1.5 Mb) (Fraser*, et al.*, 1997). Orthologous proteins of the chemotaxis regulatory system have been identified by genomic analysis in *Leptospira* spp.*,* but genetic manipulations of *Leptospira* spp. are scarce and few studies have investigated the mechanisms of chemotaxis (Ko*, et al.*, 2009).

In this study, we describe an operon containing an unusual two-component system that may be involved in the chemotaxis regulatory system of *Leptospira* spp. We used videomicroscopy and developed an algorithm to analyze the motility of *Leptospira* strains.

Material and Methods

Leptospiral strains and culture conditions. *Leptospira interrogans* serovar Manilae strain L495 was cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and on 1% agar-EMJH plates at 30°C.Spectinomycin and/or kanamycin were added to the culture medium at 50 $\mu g.ml^{-1}$ where appropriate. Growth curves were determined by the optical density at 420 nm (OD_{420}) in liquid EMJH medium at 30°C with shaking.

Genetic manipulation of *L. interrogans.* The random mutagenesis of *L. interrogans* serovar Manilae strain L495 with the transposon *Himar1* was conducted as described previously (Bourhy, *et al.*, 2005). The location of the transposon insertion was determined by semi-random PCR (Murray, *et al.*, 2009). The confirmation of genotypes of mutants was performed by PCR with primers located in the sequences flanking the insertion site of the transposon (Table 1) and Southern blots of EcoRI-digested DNA probed for hybridization with the kanamycin resistance cassette (data not shown).

Gene expression analysis. *Leptospira* strains were cultured in duplicate to a density of 2 x 10^8 bacteria per ml at 30°C with shaking. Cultures were harvested via centrifugation at 3,200 *g* and RNA was extracted with TRIzol (Invitrogen) as previously described (Eshghi, *et al.*, 2012). Synthesis of cDNA and quantitative Reverse Transcriptase-PCR (qRT-PCR) were performed as previously described (Eshghi, *et al.*, 2012) with the following modification: the normalizing gene used in the present study was *rpoB* (LA3420). The primers used for quantification are listed in Table 1.

Chemotaxis assays. Soft agar and capillary assays were performed as previously described (Lambert, *et al.*, 2012). For the capillary assay, the relative chemotactic response was calculated as: (Number of bacteria in capillary containing attractant – Number of bacteria in capillary containing buffer only)/ Number of bacteria in capillary containing buffer only.

Motility and tracking assays. Bacteria were centrifuged at 3000g for 10min and were resuspended in liquid motility buffer (Lambert, *et al.*, 2012) and in a viscous motility buffer (1% methylcellulose Merck 15cP) to study motility and chemotaxis in low and high viscous media, respectively. *L. interrogans* cells were observed by darkfield videomicroscopy (Olympus BX-53 coupled with a camera Hamamatsu Orca Flash2.8). Bacteria were diluted to obtain approximately ten bacteria per field at 20X magnification. Approximately ten movies of 2 minutes were recorded for each strain. This work was repeated in at least two independent experiments. Movies were analyzed with MATLAB 2011. After subtracting the background, images were smoothed and a boundary detection algorithm was applied. We reconstructed the individual trajectories by associating the center of mass between consecutive frames. For each trajectory, we counted the number of reversals which were defined as any change in direction of a total of 180 +/- 45 degrees that occurred within 3 time frames (0.3s). Reversal frequency was then assessed in the whole population.

Animal experiments. Leptospires were injected intraperitoneally at doses of 10^6 into groups of eight 28 day old male gerbils (Janvier®) (*L. interrogans* serovar Manilae 50% infective dose [ID_{50}] of 10 (Murray, *et al.*, 2009)), which were monitored for up to 21 days. Protocols for animal experiments were approved by the Animal Care and Use Committee of the Institut Pasteur (France). Data were plotted on a graph and were analyzed by GraphPad Prism 5.0c (GraphPad Software, San Diego, CA). Quantitative PCR (qPCR) was used to quantify bacteria in target organs. Various groups, each containing four gerbils, were infected intraperitoneally with 10^8 leptospires and were killed at day five postinfection. The liver and kidneys were collected, and approximately 50 µg of tissue was homogenized in PBS. Total genomic DNA was then extracted with a tissue DNA purification kit (Maxwell; Promega). The concentration of leptospires in tissues was quantified by a TaqMan assay targeting the *lipL32* gene (Lourdault, *et al.*, 2009), by qPCR with the CFX96 real-time PCR detection system (Bio-Rad). The standard curve for genome

equivalents per gram of tissue was determined by 10-fold serial dilutions of purified DNA of a known concentration of *L. interrogans* serovar Manilae strain L495. All PCRs were performed in duplicate and control reactions without a template were included in each assay.

RESULTS

Identification of a random mutant in a chemotaxis operon.

Analysis of the genome of the pathogen *L. interrogans* serovar Manilae strain L495 revealed the presence of genes coding for 26 chemotaxis proteins, including 12 MCPs, three CheW, two CheA, two CheR, three CheB, two CheD, and two CheY (http://mistdb.com). Similar to most bacteria, many of the chemotaxis genes are clustered in the genome of *L. interrogans* (Wadhams & Armitage, 2004). We generated a library of random transposon mutants in the strain L495 (Bourhy*, et al.*, 2005, Murray*, et al.*, 2009) and obtained one mutant, named M641, in which the transposon had inserted into the first gene (LA2421/LIC11528) of an operon-like structure. This structure contained six genes coding for putative chemotaxis proteins (CheA, CheW, CheD, CheB, CheY, and MCP) and one gene encoding a putative anti-sigma factor antagonist (Fig 1A). Interestingly the first two genes encode atypical proteins which were not previously described as associated with chemotaxis gene orthologues. The first gene, LA2421 encodes a putative histidine kinase (731 amino acids) composed of a phosphoacceptor domain of the HisKA family, an ATPase domain, and a PAS domain, which is usually a signal sensor domain in many signaling proteins. The second gene encode for the protein RR (426 amino acids) which contains an N-terminal receiver domain and a C-terminal effector domain that includes an ATPase of the GHKL family. The structure/function of the central domain (≈150 amino acids) of RR is unknown. Of note, we did not identify a DNA-binding domain such as a helix-turn-helix motif in this protein. RR therefore appears to be an atypical response regulator.

The locus is composed of a contiguous stretch of nine genes oriented in the same direction, suggesting that they may be co-transcribed in an operon. We used reverse transcriptase PCR (RT-PCR) and primers corresponding to specific sequences within pairs of adjacent genes to confirm the existence of an operon experimentally. We detected transcripts that overlapped adjacent genes all along the locus (LA2421-LA2422, LA2422-LA2423, LA2423-LA2424,

LA2424-LA2425, LA2425-LA2426, LA2426-LA2427, LA2427-LA2428, LA2428-LA2429) (data not shown). We did not detect transcripts in genes downstream of the inactivated genes LA2421 and LA2422 (Fig. 1), further indicating that the nine genes of this locus constitute an operon.

Homologs of the genes of this operon are present in all *Leptospira* species, including the saprophyte *L. biflexa* and the intermediate *L. licerasiae*. However, LA2421 and LA2422 genes do not appear to co-localize with chemotaxis genes in any other bacteria. We subsequently designated the *L. interrogans* serovar Manilae strain that had a transposon insertion in LA2421, mutant *HK*.

The *HK* mutant has altered chemotactic behavior

The *HK* mutant exhibited normal growth at 30°C (data not shown). We performed both soft agar and capillary assays to evaluate the chemotactic behavior of the mutant (Lambert*, et al.*, 2012). In soft agar, either at 0.5% (Fig 2A) or at 0.3% (Fig 2B), HK mutant formed significantly smaller colonies than the wild-type strain. This result suggested a default in chemotaxis and/or motility. We used a capillary assay with Tween80 as a chemoattractant to characterize further chemotaxis (Lambert*, et al.*, 2012). The response of HK mutant to Tween 80 at 0.5% was significantly different from that of the wild-type strain (Fig 2C.). Thus, this mutant demonstrates altered chemotactic behavior that may be attributed to chemotaxis deficiency or altered motility.

We sought to identify the cause of altered chemotactic behavior of these mutants. We developed a method to assess the motility of *Leptospira* spp. in liquid media by videomicroscopy. We assessed the speed of bacteria from more than 100 trajectories of each strain that were visualized by videomicroscopy in either liquid media or in viscous media, to characterize the motility and chemotaxis of the mutant.

Mutant *HK* shows impaired reversal ability.

Unexpectedly, there was no significant difference in the speed and the trajectories of the *HK* mutant and the wild-type strains in low (data not shown) and high viscous media (Fig. 3 and 4A). The altered chemotaxis motility of the *HK* mutant in soft agar and capillary assays prompted us to investigate in more detail its motility. We defined an algorithm to determine reversal frequency. Reversal is a switch of direction of the swimming bacteria.

We counted the number of reversals for each trajectory. Reversal was defined as a 180 +/- 45 degrees change in direction that occurred within three time frames (0.3s) (Figure 4 B,C). We then calculated reversal frequency in the whole population. The reversal frequency was significantly lower in the *HK* mutant than in the wild-type strain (Fig 4D). However, mutant was still able to reverse, showing that the flagellar motors could rotate in both directions. These data suggest that mutation in the gene encoding the histidine kinase affects the chemotaxis system which then results in an altered motility behavior. Interestingly, altered motility could not be observed from the previously measured variables (speed and trajectory shape). This observation reinforces the utility of developing a videomicroscopy approach.

The HK mutant is virulent.

We tested the virulence of the *HK* mutant by intraperitoneal infection of gerbils with 10^6 bacteria (Fig. 5). The wild type and the *HK* mutant caused death 5 to 6 days after infection. We assessed bacterial burden by qPCR in the kidneys and liver from gerbils to investigate the effect of the *HK* mutation on dissemination *in vivo*. Groups of four gerbils infected intraperitoneally with 10^6 bacteria (either wild-type or *HK* mutant strains) were killed 5 days postinoculation (before the first infection-related death among animals infected with the wild-type or *HK* strains) (Fig. 5). Bacterial burden in tissues of animals infected with the *HK* mutant was similar to that of tissues from animals infected with the wild-type strain.

DISCUSSION

Chemotaxis in *Leptospira* spp. and its role during the infection process remains largely unexplored. In this study, we obtained a mutant in a chemotaxis operon of the pathogen *L. interrogans.* This operon contains the chemotaxis orthologous proteins CheA, CheW, CheD, CheB, CheY, and MCP. CheW links the membrane sensor MCP to the kinase CheA, which autophosphorylates and transfers the phosphoryl group to either CheY or CheB. CheB and CheD are responsible for the methylation status of MCP. CheY interacts directly with the flagellar switch, regulating the sense of rotation of the flagellar motor. No information is available about the role of each chemotaxis protein in *Leptospira* spp., with the exception of data from studies involving heterologous complementation in *E. coli che* mutants (Li*, et al.*, 2006, Li*, et al.*, 2006, Zhang*, et al.*, 2013). Chemical attractants have been identified in *Leptospira* spp. (Yuri*, et al.*, 1993, Lambert*, et al.*, 2012), but the specificity of each chemotaxis receptor protein (MCP) for these attractants is not known.

In this study, we analyzed one mutant in which a transposon was inserted in the first gene of a chemotaxis operon, which codes for a putative histidine kinase. This protein has never been described as involved in chemotaxis and this gene is not genetically linked to any chemotaxis genes, except in *Leptospira* spp. Because most genes of the locus code for chemotaxis protein, we hypothesized that LA2421 and LA2422 encode components of the chemotaxis signal transduction system in *Leptospira* spp.

The mutant was deficient in chemotaxis, which supports the hypothesis that the operon is involved in chemotaxis. The *HK* mutant was virulent and had translational movements but slow reversal. The altered motile behavior of the *HK* mutant may not affect its virulence if injected intraperitoneally. Other routes of infection should be tested to determine if lack of reversal is

important in the early steps of the infectious process. Studies have shown that mutants *cheB* (LA1252) and *cheX* (LA2469) in *L. interrogans* serovar Manilae retain their virulence (Murray*, et al.*, 2009). We also tested transposon mutants in genes encoding MCPs (LA2246, LA0676, LA0678, LA1214, LA1191, and LA0070); virulence was not attenuated in these mutants (unpublished data). This suggests functional redundancy within members of this protein family.

In summary, we show that the *HK* mutant is virulent, with a reversal frequency lowered compared to WT strain, deficient in chemotaxis, and motile in contrast to the avirulent *flaA2* mutant which lacks functional flagella (Lambert*, et al.*, 2012). Thus, our findings advance one step further the understanding of the role of motility during the pathogenic process.

Acknowledgments

The authors thank Massimo Vergassola, Eloise Bertiaux, Nadia Benaroudj, and Alejandro Buschiazzo for their support and assistance. This work was supported by the Institut Pasteur and the French Ministry of Research (ANR-08-MIE-018). This work is part of the doctoral program of the University Paris Diderot of A. Lambert.

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Figure legends

FIG 1 Chemotaxis operon of *L. interrogans*.

A) Genetic organization of the locus encoding numerous chemotaxis orthologous genes and insertion sites of the transposon in *HK* and *RR* mutants (black arrows). B) Quantification of selected genes in the above described genetic locus via RT-PCR in the *HK* mutant relative to Wild-Type (Fold Change). Results of at least two independent experiments are shown.

FIG 2 Analysis of bacterial motility and chemotaxis.

EMJH plates containing 0.3% or 0.5% agar were inoculated with the wild-type, *HK* mutant strain. Colony diameter was measured after 19 days (A) 0.5% Agar, (B) 0.3% Agar. Data representative of three independent experiments are shown. (C) WT and *HK* mutant strain were analyzed by capillary assay (C). Data are representative of three independent experiments (*** : significant difference between mutant and WT, with a p value < 0.0001).

FIG 3 Analysis of the trajectories of WT, and *HK* mutant strains.

Plot of trajectories collected from over 30 movies of two minutes in three independent experiments. Analysis were performed in viscous buffer containing 1% methylcellulose.

FIG 4 Speed and reversal frequencies analysis of *L. interrogans* strains

(A) Histogram showing the speed of trajectories analyzed for the two strains in viscous media.

(B) Plot of one bacterial trajectory (blue line). Red circles show reversal events

(C) Speed (red) and angle change (blue) for the same trajectory. Detected reversals are shown by a green cross.

(D) Histogram showing the reversal frequencies in viscous media calculated from the trajectories of *HK* and WT strains

FIG 5 Inactivation of *HK* do not alter virulence in *L. interrogans*.

(A) Survival curve of infected gerbils. Groups of eight animals were injected intraperitoneally with 10^6 leptospires of *L. interrogans* WT or *HK* strains. (B) Bacterial burden of organs from gerbils after infection with 10^6 leptospires. The organs (K, kidney; L, liver) of animals were recovered 5 days postinoculation, genomic DNA was extracted, and real-time PCR was carried out. Geq, genome equivalent; *, not detected (threshold cycle [CT] values of *40).

Supplementary data

Table S1: List of primers used in this study

Movie S1 Movie (X100, darkfield hamamatsu 20fps, 51 sec) WT motility in methylcellulose 1% viscous motility buffer

Movie S2 Movie (X100, darkfield hamamatsu 20fps, 23 sec) HK motility in methylcellulose 1% viscous motility buffer