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"Role of the *R. inulinivorans* flagellum in adherence to intestinal constituents and gut colonization"

Fernando Javier Díaz Yáñez.

Thesis director: Dr. Fernando Rafael Gil Michell.

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En Santiago, el día 20 enero de 2023, en ceremonia solemne se efectúa la defensa pública de la Tesis presentada por el **Sr. Fernando Javier Díaz Yáñez "Role of the *R. inulinivorans* flagellum in adherence to intestinal constituents and gut colonization"** como el último requisito para la obtención del grado de Doctor en Biotecnología de la Universidad Andrés Bello. La comisión examinadora está integrada por su Director de Tesis Dr. Fernando Gil, además de los Doctores Daniel Paredes, Juan Fuentes y Roberto Vidal.

Esta ceremonia es co-presidida por la Vicerrectora de Investigación y Doctorado, Doctora Carolina Torrealba y por el Decano de la Facultad de Ciencias de la Vida, Doctor Alfredo Molina y la Directora Académica de Doctorados, Doctora Erika Poblete.

Luego de escuchar la defensa de la Tesis expuesta por el Sr. Fernando Javier Díaz Yáñez, la comisión ha decidido aprobarla y manifestar sus felicitaciones.

Por lo anterior, se otorga el grado de

Doctor en Biotecnología

A

Fernando Javier Díaz Yáñez

Dr. Fernando Gil
Director de tesis
Universidad Andrés Bello

Dr. Daniel Paredes
Evaluador Externo
Texas A&M University

Dr. Juan Fuentes
Integrante Comisión
Universidad Andrés Bello

Dr. Roberto Vidal
Evaluador Externo
Universidad de Chile

Dr. Juan Fuentes
Ministro de Fe
Universidad Andrés Bello

THESIS DEDICATION

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"Deberías disfrutar de los pequeños desvíos,
disfrútalos mucho,
porque de ese modo encontrarás aquello
que es más importante que lo que querías"

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LIST OF ABBREVIATIONS

- CCW = Counterclockwise
- CW = Clockwise
- EMS = Ethyl metanesulfonate
- FBB = Flagellum basal body
- Fla^- = Non-flagellated mutant
- Fla^--TC = Transconjugant non-flagellated mutant
- FM = Flagellar-motility
- GF = Germ-free
- GIT = Gastrointestinal tract
- MI/GM = Microbiota intestinal/Gut microbiota
- OD_{570} = Optical density at 570 nm
- PBS = Phosphate-buffered saline
- rFla+ = Revertant
- sDMEM = DMEM medium supplemented with 10% of fetal bovine serum
- t_d = Doubling time
- TEM = Transmission electron microscopy
- WT = Wild-type
- WT-TC = Wild-type complemented
- YCFA-CGM = YCFA medium supplemented with cellobiose, glucose, and maltose

1. RESUMEN.

La microbiota intestinal (MI) humana, benéfica para la salud del ser humano, está dominada en su mayoría por dos filos bacterianos, Bacteroidetes y Firmicutes. La colonización intestinal por parte de estas bacterias es un proceso multifactorial en los que están implicados, entre otros, la motilidad y la adherencia a componentes del intestino. La motilidad mediada por flagelos cumple un rol fundamental en la colonización y virulencia de múltiples bacterias patógenas, sin embargo, la importancia de la motilidad flagelar en la colonización de bacterias comensales de la MI, en particular del filo Firmicutes, ha sido pobremente estudiada. El flagelo permite el movimiento de manera concertada en búsqueda de condiciones favorables en medios líquidos y semi sólidos, pero además actúa como adhesina a componentes del intestino como la mucina y células epiteliales. *Roseburia inulinivorans* (*R. inulinivorans*) es una bacteria de la microbiota intestinal de diferentes organismos, flagelada y que pertenece a un género importante de bacterias en la microbiota intestinal. Los determinantes genéticos que participan en la colonización de *R. inulinivorans* no han sido descritos, ya que no existen herramientas de manipulación genética sitio dirigidas en este género, aunque *R.* inulinivorans ha podido ser conjugada con plásmidos derivados de la línea pMTL, convirtiéndola en un buen candidato para la búsqueda de determinantes genéticos relacionados a la colonización de este género. Mediante mutagénesis química obtuvimos mutantes sin flagelo de *R. inulinivorans* (Fla^-), que posterior a la secuenciación del genoma completo de estas pudimos observar que las mutaciones generadas afectaban a las proteínas FlhB, FliM y FliP, del cuerpo basal del flagelo. Estas mutantes *in vitro* poseen una mayor formación de biopelículas y adherencia a células epiteliales, y por el contrario una menor adherencia a una capa de mucina. Las revertantes espontaneas generadas para cada mutante Fla^- ($rFla^+$) confirmaron que los fenotipos observados se produjeron por las mutaciones en las proteínas descritas previamente, excepto en FlhB. Interesantemente, las cepas revertantes poseen una mayor motilidad que la cepa silvestre, lo que podría ser explicado por mutaciones en las proteínas FliF o CheY, que forman en anillo MS del cuerpo basal o participan la regulación de la quimiotaxis, respectivamente. En la colonización de ratones no observamos diferencias significativas entre las cepas mutantes y silvestre, aunque desde las heces solo recuperamos cepas Fla^-. Por lo tanto, los genes *fliM* y *fliP* son funcionales y necesarios en el ensamble del flagelo en *R. inulinivorans* y, además, la pérdida de este afecta de forma contraria la adherencia a las células epiteliales y mucina. Además, si bien no obtuvimos diferencias significativas en la colonización, observamos una tendencia que indicaría que en ausencia del flagelo *R. inulinivorans* favorecería la colonización intestinal.

2. ABSTRACT.

The human gut microbiota (GM), essential for human health, is dominated by two bacterial phyla, Bacteroidetes and Firmicutes. The intestinal colonization of these bacteria is a multifactorial process involving motility and adherence to intestinal components. Flagellar-motility (FM) plays a vital role in the colonization and virulence of multiple pathogenic bacteria. However, FM's in colonizing commensal bacteria from IM, particularly in the Firmicutes phylum, has been poorly studied. Flagellum all the motility in a concerted way in liquid and semi-solid environments for the search of more favorable conditions but also participates as an adhesin to intestinal components, such as epithelial cells and mucin. *Roseburia inulinivorans* (*R. inulinivorans*), is a flagellated member of the IM in different organisms that belongs to an important genus of the GM. Genetics determinants that participate in *R. inulinivorans* have not been determined yet due to the lack of site-directed mutagenesis tools for this genus. However, *R. inulinivorans* has been conjugated with pMTL-derived plasmids, which gives this bacterium an advantage over the rest of this genus in searching for genetic determinants related to intestinal colonization. Using chemical mutagenesis, we obtained non-flagellated mutant strains of *R. inulinivorans* (Fla^-), which were analyzed by whole genome sequencing (WGS) determining that present mutations on FlhB, FliM, and FliP proteins. Fla^- mutants *in vitro* have a higher biofilm formation and adherence to epithelial cells, and lower adherence to mucin when compared to the wild-type (WT) strain. Spontaneous flagellated revertant ($rFla^+$) strains obtained for each mutant confirm that mutations on each Fla^- mutants generate the non-flagellated phenotype, except for FlhB. Interestingly, $rFla^+$ strains have higher motility than WT strains which can be explained by mutations found on FliF or CheY, the MS-ring, or the chemotactic regulatory protein, respectively. We have not observed significant differences between Fla^- mutants and WT strain in mice colonization, although we only obtain Fla^- mutants and no WT strain bacteria from feces. Therefore, *fliM* and *fliP* are functional genes necessary for flagellum assembly in *R. inulinivorans*, and the loss of this structure affects in a converse way the adherence to epithelial and mucin. Also, even when we did not obtain significant differences in mice colonization, we observed a tendency that could indicate that flagellum absence favors intestinal colonization in *R. inulinivorans*.

3. INTRODUCCIÓN.

The gut microbiota (GM) term includes all the microorganisms that inhabit the intestinal tract. These are archaea, viruses, fungi, and bacteria; together the GM plays a crucial role in the health and wellness of the host including nutrient extraction, synthesis, and absorption; defense against pathogenic microorganisms; development and maintenance of the immune system; among others (Rinninella et al., 2019). Bacteria are the most considerable weight of biological mass in the intestine. Most of these bacteria belong to five phyla and, more specifically to one thousand species. Two important phyla of the GM are Bacteroidetes and Firmicutes are more than 90% of the bacteria in the intestine (Sekirov, Russell, Antunes, & Finlay, 2010). Commensal bacteria of the Firmicutes phylum are poorly studied in their colonization processes and factors due to the lack of genetic tools to manipulate them, even when some of them are highly represented in the intestine as *Faecalibacterium prausnitzii* and *Roseburia spp.* (Aminov et al., 2006) Bacterial populations are highly determined by the environmental characteristics which could select the growth of determined groups of bacteria. In the gastrointestinal tract (GIT) the colon has the highest abundance of bacteria with trillions of these microorganisms, which could grow in the lumen or the epithelium surface.(Rinninella et al., 2019)

3.1. The colon: a niche for bacteria.

The GIT in a healthy adult human is nine meters long and includes the mouth, esophagus, stomach, small intestine, and large intestine or colon. The transit time along the GIT has been estimated at approximately 30 h, where the colon is the slowest zone with 23h of transit (Procházková et al., 2022). Each zone of the GIT has characteristics that favor the growth of determined groups of bacteria over others. For example, in the mouth, there are a high number of bacteria; 10^{11} bacteria/g in dental plaque and $10^{11\text{-}12}$ bacteria/mL in saliva, which diverge in composition on each mouth surface due to the surface differences present there. The esophagus has a mucosal surface considered free of colonizing bacteria, and the bacteria present here come from the mouth. In the stomach, its low pH favors the growth and colonization of acidophilic bacteria. Moreover, this adverse characteristic causes the lowest abundance of bacteria in the GIT, $10^{3\text{-}4}$ bacteria/g of wet weight; and the microorganism in this zone colonize the mucosal surfaces. The small intestine is the most considerable portion of the GIT, here the pH increases due to pancreatic enzymes and bile salts which let more bacteria colonize and grow, in this zone bacteria/g of wet weight increases from the proximal

to the distal small intestine; from 10^{3-4} to 10^{7-8} bacteria/g of wet weight; bacteria colonize the lumen and the mucosal barrier where bacteria face different surfaces as epithelial cells and a mucus layer similar to the stomach and colon. Finally, in the colon, bacteria can grow in the lumen or the mucosal barrier; here the epithelial cells are covered by a mucus barrier that avoids the attachment and growth of bacteria close to the epithelial barrier of the mucosa. As a result, the colon has the highest abundance and diversity of the GIT, 10^{10-11} bacteria/g of wet weight, and bacteria that live in the lumen or the mucus layer belong to varied species and phyla (Rinninella et al., 2019).

3.1.1. Colon characteristics.

The colon is the final portion of the GIT and plays three important roles: water and electrolytes absorption, vitamin production and absorption, and forming and propelling feces for material elimination.(Bharucha & Camilleri, 2019) The large intestine is approximately 1.5 m large and 7.5 cm wide in healthy adults, it has a slightly acidic pH from 5.5 in the proximal part, progressively increasing to 6.7 in the distal colon. Other characteristics are the reduced oxygen availability, which is perfect for the growth of anaerobic bacteria, the oxygen diminishes from the esophagus to the final portion of the colon, and the same occurs with antimicrobial peptides that can explain the increase of the bacterial abundance when it is compared with the rest of the GIT portions (Donaldson, Lee, & Mazmanian, 2016).

In the colon, a continuous mucus layer covers the epithelial barrier of the mucosa, and this mucus layer is divided into the inner and outer mucus layers. The mucosa of the large intestine is formed by an epithelial layer that creates a surface punctuated by invaginations known as colonic crypts, these crypts are like tubes with a central hole that forms the crypt lumen where absorption of nutrients occurs (Johansson, Larsson, & Hansson, 2011).

3.1.2. The mucus layers.

The colonic mucus layer is conformed principally by mucins that are large, highly glycosylated proteins secreted by goblet cells in the colonic crypts. Mucin structure is more than 80% carbohydrates that cover a protein core rich in proline, serine, and threonine amino acids. The last two amino acids supply an attachment site in their hydroxyl groups for N-acetylgalactosamine that are added inside the Golgi apparatus by glycosyltransferases. Next, other glycosyltransferases add new residues from the N-acetyl galactosamine residues, extending and branching the mucin structure, generating a complex mixture of glycan

epitopes. There are at least twenty-one genes coding for mucin proteins, but Muc2 is the principal component of the colonic mucus barrier in both the inner and outer layers, mixed with many other proteins. There are differences between both layers, principally the inner layer is thinner and denser than the outer layer with no colonizing bacteria, instead, the outer layer is used as a niche for many bacteria. Another characteristic that differentiates between both layers is that the inner layer is firmly attached to the epithelium while the outer layer is loose and easily removable (Johansson et al., 2011). The mucus layer is a gel-like matrix so bacteria can move inside to find nutrients and growth, and some bacteria can even use the mucin glycans as a nutrient (Huang, Lee, & Mazmanian, 2011).

3.2. Colonization factors: Flagella relevance.

Several colonization factors have been found in bacteria, contributing directly or indirectly to colonization. Among important colonization factors are adhesins structures and proteins, motility, bacteriocin production, and metabolic routes that improve fitness; these factors are used for bacteria to compete, colonize and/or persist in the niches (Donaldson et al., 2016; Kommineni et al., 2015). To colonize the GIT, bacteria must overcome different adverse conditions and the colonization resistance of the microbiota. One structure that takes part in two of the factors mentioned above is the flagellum, a complex structure that allows motility but also functions as an adhesin (Haiko & Westerlund-Wikström, 2013; Liu & Ochman, 2007)

3.2.1. Adhesins as colonization factor in GIT.

Bacterial adherence is the link between a bacterium and a substrate, where separating both requires energy. For the colonization of GIT, adhesion to the mucosa is considered a prerequisite and is relevant for both pathogenic and commensal bacteria. In the GIT the adhesion to the epithelial barrier requires an adhesin in the bacteria and a receptor in the host surfaces; the last could be cellular membrane constituent or associated with these, and can be categorized into three classes: integrals, peripheral or cell surface coat components (Hao and Lee, 2020).

Integral cellular membrane constituents acting as receptors are glycolipids and glycoproteins, while peripheral constituents are proteins or glycoproteins anchored to the cellular membrane by weak ionic interactions or hydrogen bonding. An example of these peripheral constituents is fibronectin, a substrate for bacterial adhesins. Finally, the surface

coat constituents are high molecular weight glycoproteins, the mucins of the mucus layers (Hao & Lee, 2020). On the other hand, adhesins are bacterial proteins that can recognize specific carbohydrate sequences in glycolipids and glycoproteins or defined protein structures. In Gram-negative bacteria, adhesins are principally in structures such as fimbriae and pili, while in Gram-positive bacteria are usually in the cell wall. In both, the flagellum acts as an adhesin and allows the linkage to intestinal components (Hao & Lee, 2020).

3.2.2. Bacterial flagellum: Structure and assembly.

The flagellum is a complex structure made up of three well-defined significant parts, the filament, hook, and basal body (FBB) that in turn, together, are made up of twenty to thirty different proteins (Liu & Ochman, 2007). The filament allows motility when rotating, and this rotation is generated by the FBB and communicated through the rod and hook. The quantity and arrangement of flagella depend on the bacteria phyla, genre, and/or species; in this manner can be bacteria with one or more flagellum per cell and the distribution could be varied. Depending on these, bacteria are monotrichous or lophotrichous when having one or more flagella at one end of the bacterium, respectively. If a bacterium has one or more flagella at both ends is considered amphitric, and when the flagella are distributed throughout the bacterium is considered peritrichous (Rajagopala et al., 2007)

Most flagellar proteins are highly conserved in bacteria and only a few genes are genus or species-specific, the same occurs with accessory genes related to motility as chemotaxis, such as CheA and **CheY**, and regulatory genes (Rajagopala et al., 2007). In Gram-Positive bacteria, the flagellum assembly starts with the MS-ring formation which is formed by **FliF** protein when it is incorporated into the cytoplasmatic membrane. Next, the stator of the system which is composed of MotA and MotB encompasses the MS-ring, followed by the assembly of a flagellum-specific export apparatus that is a type 3 secretion system composed of nine transmembrane proteins, FlhA, **FlhB**, FliH, FliI, FliJ, FliO, **FliP**, FliQ, and FliR. After, **FliM** and FliN attach to the MS ring by the cytoplasmatic face through FliG forming the C-Ring that covers the f-T3SS (in bold are important genes for this thesis). Together, the MS-ring, the stator proteins, the C-ring, and the export apparatus form the FBB, that are responsible not only for flagellum rotation, but also the exportation of the proteins of the rod, the hook, and the filament. When the FBB is formed, the assembly of the rod start inside of it, the structural proteins of the rod are FliE, FlgB, FlgC, FlgF, and FlgG, which form a channel. Next, proteins of the hook, FlgE, FlgD, and FliK, are transported through the rod to start with the hook formation, a process that ends when FlgK and FlgL, two proteins that take part in

the hook-filament junction, join to the distal hook. Finally, FliD forms the filament cap, and the polymerization of FliC produces the filament elongation, where each subunit of flagellin (FliC) is inserted between FlgL and FliD (Apel & Surette, 2008). The flagellum formation follows the sequential steps described above (Figure 1) and takes 20 minutes to complete. Specific mutations have proved that some of these proteins are needed for the assembly or the correct functioning of the flagellum. For example, some mutations in the FBB or the filament proteins avoid the flagellum assembly generating non-flagellated strains, while other mutations produce flagellated strains but with paralyzed flagella, such as mutations in the stator component (Cheng et al., 2018; Garza, Biran, Wohlschlegel, & Manson, 1996). These studies have also contributed to the study of flagellum relevance in the colonization process.

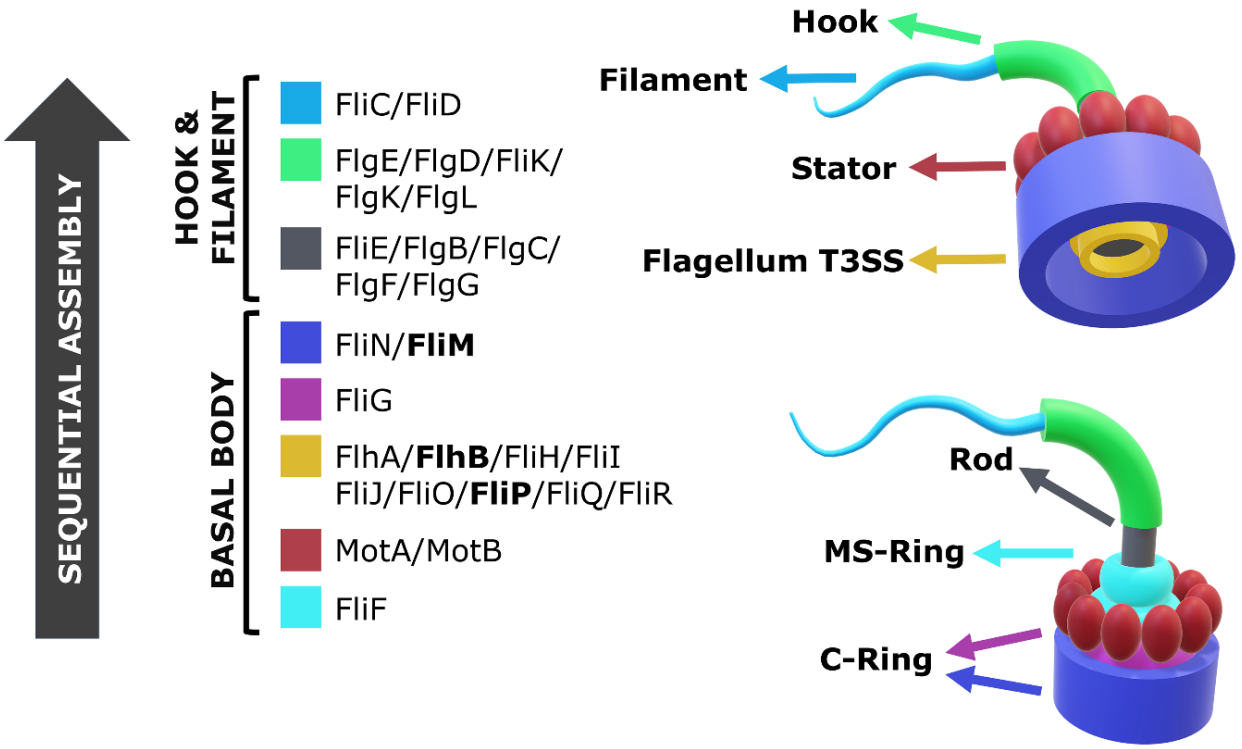


Figure 1. Flagellum structure and sequential assembly in Gram-positive bacteria. The flagellum is a complex structure with three major parts, the basal body, the hook, and the filament. Between 20 to 30 proteins form the flagellum, depending on the species. The assembly starts with the basal body's MS-ring (light blue) formation in the cytoplasmatic membrane and the stator proteins (red) surrounding it. This is followed by the formation of the flagellum exporting apparatus (yellow), a flagellum-specific type 3 secretion system in the cytoplasmatic face of the MS-ring. Next, the C ring (blue and violet) joins the MS ring to complete the basal body structure. After, through the exporting apparatus, the proteins of the rod (grey) are exported from the cytoplasm to the periplasmatic space and assembled crossing

the cell wall and forming a channel that allows the transporting of the proteins that form the hook (green) and, finally, the filament (pale blue). Words in bold are genes relevant to this thesis.

3.2.3. Bacterial flagellum in the gut colonization process.

The principal function of the flagellum is motility, the ability to move in an independent and concerted manner in aqueous or semi-solid environments, in the gut represented by the lumen and mucin layer, respectively (Haiko & Westerlund-Wikström, 2013). The motility allows bacteria to move to the nutrients or get to specific places in the gut, as the epithelial barriers under the mucin layers. Also, flagellum can function as an adhesin, as mentioned before, where both filament proteins, FliC and FliD, have shown the capability to function as an adhesin in many bacteria species (Baban et al., 2013; Girón, Torres, Freer, & Kaper, 2002; Rabaan, Gryllos, Tomás, & Shaw, 2001). Motility and adherence are independent, opposite but complementary processes, while flagellum lets bacteria reach the colonization site, and in some bacterial species it could take part in the primary adherence, motility must be downregulated and expression of adhesins must increase for an adequate adhesion.

To determine if a structure or protein act as adhesin is challenging to evaluate *in vivo* because could be taking part in one or more processes, such as the flagellum that participates as adhesin and propulsor. Furthermore, evaluating if a simple structure or protein can contribute to gut adhesion is difficult because many factors could contribute to this phenomenon. Usually, to study flagellum as an adhesin to intestinal components, mutants on *in vitro* models have been used, these models include monolayers of epithelial cell lines and matrix that simulate the mucus layers of the gut (McKee, Aleksanyan, Garrett, & Tamayo, 2018; Van den Abbeele et al., 2009). Both models have demonstrated that flagellum takes part as an adhesin, principally for pathogenic bacteria (Girón et al., 2002; Haiko & Westerlund-Wikström, 2013).

The study of flagellum relevance in the gut colonization process has been mainly studied in pathogenic bacteria using *in vivo* models. Evidence has shown that, for some species, the flagellum is required for colonization, while in other bacteria flagellum is not required (Baban et al., 2013; Terashima, Kojima, & Homma, 2008). In the gut of healthy humans, where only commensal bacteria are present, there is a low amount of flagellin, even though many species of the microbiota can generate flagella. This phenomenon can be explained based on the immune response, where the immune system, via Toll-like receptors, recognizes the flagellin and generate specific antibodies (Koch & Barton, 2013). It has been

shown that the joining of these antibodies to flagellin generates a decrease in the expression of genes related to motility in the bacterium (Cullender et al., 2013); moreover, motility is inhibited in many species (Landsperger et al., 1994; Riazi et al., 2013), including gut microbiota species as *Roseburia intestinalis* (Cullender et al., 2013). Based on these, Koch & Barton (2013) propose that, if flagellar motility may be inhibited during colonization, it may only be necessary for the fitness of certain species that colonize the intestine in early stages (*i.e.* after birth), or in processes of change in the intestine, which could be related to persistence. Concordantly, many bacteria that are part of the microbiota do have not flagella, such as *Faecalibacterium prausnitzii*, an important and abundant specie of the gut microbiota (Lopez-Siles et al., 2016). Because the flagellum is not a factor in the colonization of all species and considering that the expression of the flagellar machinery could be inhibited during colonization, it is crucial to assess whether the flagellum plays a role in the colonization of a particular genus or specie, even more in important genus or species of the gut microbiota, as *Roseburia*.

3.3. The Roseburia genus.

The first bacterium of the *Roseburia* genus was discovered in 1983, *Roseburia cecicola* (*R. cecicola*) was reported as part of the intestinal microbiota of mice (Stanton & Savage, 1983b). In the early 2000s four species that compose this genus, *R. faecis*, *R. hominis*, *R. intestinalis,* and *R. inulinivorans*, were found in the human intestinal microbiota (Duncan, 2002, 2006). Lately, a new species found in pig GM has been added to this genus, *R. porcis* (Wylensek et al., 2020), some other species from the microbiota have been related to this genus, such as *Agathobacter rectalis* (*A. rectalis*), a bacteria that forms part of *Roseburia spp./A. rectalis* group (Rosero et al., 2016). The *Roseburia* genus (family Lachnospiraceae, phylum Firmicutes) is characterized by being a slightly curved bacillus, Gram-variable, strictly anaerobic, non-sporulating, and motile by multiple subterminal flagella. Another characteristic is the ability to synthesize short-chain fatty acids (SCFA), such as butyrate, which is vital for human health (Duncan, 2006).

3.3.1. *Roseburia* in gut microbiota.

It is not well defined when the *Roseburia* genus colonizes the human intestine. Some studies have shown that it can be detected in newborns at low levels 72 h postpartum (Chang, Shin, Chun, Lee, & Seo, 2011), while others have demonstrated that the *Roseburia* genus is

not present during the first three months of life, despite some studies have found the presence of this genus in breast milk (Jost, Lacroix, Braegger, & Chassard, 2013; Jost, Lacroix, Braegger, Rochat, & Chassard, 2014), a possible transmission route for non-spore former anaerobic bacteria groups that has been discussed lately, where some evidence make possible the existence of an entero-mammary pathway (Lyons, Ryan, Dempsey, Ross, & Stanton, 2020). In the healthy adult, on the other hand, the abundance of the *Roseburia*/*A. rectalis* accounts for between 3 to 15% of the total bacteria in feces, making them an important group of the gut microbiota (Aminov et al., 2006). The importance of this genus is reaffirmed by the fact that a decrease in the abundance of *Roseburia* has been related to many diseases but more commonly in patients with inflammatory bowel diseases, which could be due to a reduction in the production of butyrate, an SCFA that contributes to the decrease of intestinal inflammation (Chen et al., 2014; He et al., 2019; Tamanai-Shacoori et al., 2017). Due to the importance of this bacterial genus, knowing the genetic determinants that take part in its colonization is relevant to finding ways to favor the presence of these bacteria in our microbiota.

There is no site-directed mutagenesis developed for *the Roseburia* genus yet. In despite of this, *R. inulinivorans* is the only specie that has been conjugated with expression vectors derived from pMTL plasmids (Sheridan et al., 2019), which makes *R. inulinivorans* the best candidate to do genetic studies and evaluate genetic determinants that participate or favor its colonization. One of the strategies to study these determinants is coupling chemical mutagenesis, for example with ethyl methanesulfonate (EMS), phenotypic selection, suppression analysis, and genomic sequencing-based mutational mapping, as performed for another member of the Firmicutes phylum (Bae, Mueller, Wong, Rawls, & Valdivia, 2016). *R. inulinivorans* shares the characteristics mentioned previously for the genus but differs in the use of inulin as a carbon source using a glycoside hydrolase that is not shared with the other species of the *Roseburia* genus (Hillman et al., 2020).

3.3.2. Flagellar machinery in the intestinal colonization of *Roseburia*

In *Roseburia* the flagellar machinery and related genes are principally coded in three or four loci and are highly conserved between species with few differences, for example, *R. inulinivorans* has six flagellin copies scattered throughout the genome while the other species have four (Sheridan, 2014). The Flagellum of *Roseburia* genus is composed of twenty-nine different proteins, most of them represented in figure 1, and its expression is under the control

of the sigma factor σ^{28}, except for some flagellin genes that are continuously being expressed because are under the control of a housekeeping sigma factor, σ^{43} (Neville et al., 2013).

It has not been previously determined whether *Roseburia* flagellum could contribute to the adhesion to intestinal components, such as epithelial cells or mucus layer. However, the Lachnospiraceae family, a dominant family in murine models, can colonize the intestine mucosa, including the mucus layer (Li et al., 2015). Then, members of this family as *Roseburia* should interact previously with these components to generate effective colonization. Consistently, *R. intestinalis* has shown the capability to adhere to the mucus layer in an *in vitro* model of the intestinal environment. Also, this study reported that the *Roseburia* genus dominates the abundance of bacteria in this layer using a mix of bacteria obtained from fecal samples (Van den Abbeele et al., 2013). These suggest that, *in vitro*, *Roseburia* can penetrate the mucus layer matrix by an undetermined mechanism, where flagellum might be relevant because it allows bacteria to penetrate and move within gel-like media such as the mucus layer.

The first study where the absence of the flagellum on the colonization of the *Roseburia* genus was conducted in mutants of *R. cecicola* without flagellum or with defects in motility, which were generated by aleatory mutagenesis using UV light but genes responsible for these phenotypes were no determined. In that study, the flagellum, through motility, is not a key factor for colonizing of the cecum of Germ-Free (GF) mice. However, when *R. cecicola* competes for the niche with other species for colonization of the caecum (GF mice colonized with five distinct species after *R. cecicola* inoculation), the flagellum and motility are essential (Stanton & Savage, 1983a). Even though motility is not necessary for the colonization of the caecum in GF mice for one species of *Roseburia*, in another study it was observed that *R. hominis* expresses genes associated with flagellar motility when it is in the intestine of this type of mice, which was also verified at the protein level when evaluating the expression of flagellin (Patterson et al., 2017). These extremely *in vivo* controlled studies gave essential information about the colonization process when a null or limited quantity/diversity of bacteria is present in the intestine, such as in the early stages of development. Nevertheless understanding whether flagellum could contribute to the colonization of *Roseburia* in normal conditions (*i. e.* individuals with normal microbiota development) is yet to be studied.

Based on this background, we have asked if the flagellum of *R. inulinivorans* allows the bacterium to adhere to intestinal components *in vitro* and whether it contributes to intestinal colonization in conventionally-raised mice.

4. HYPOTHESIS.

Flagellum of *R. inulinivorans* is required for intestinal constituent adherence *in vitro* and gut colonization in a murine model.

5. GENERAL OBJECTIVE

To determine the flagellum relevance in the adherence to intestinal constituents *in vitro* and in the intestinal colonization *in vivo* in a murine model.

6. SPECIFIC OBJECTIVES

6.1. To evaluate the conditions for the generation of non-flagellated mutant strains.

- 6.1.1. To determine EMS concentration for random mutagenesis in *R. inulinivorans.*
- 6.1.2. To generate non-motile mutants by random mutagenesis.
- 6.1.3. To generate spontaneous revertant strains from the no-motile mutant.

6.2. To characterize phenotypically and genetically the mutants and revertant strains.

- 6.2.1. To determine flagella presence in no-motile mutants and revertant strains.
- 6.2.2. To determine motility differences between wild-type, mutants, and revertant strains.
- 6.2.3. To determine mutations generated during random mutagenesis and spontaneous reversion.

6.3. To assess the effect of flagellum loss in adherence to intestinal components *in vitro* and gut colonization *in vivo*.

- 6.3.1. To evaluate the adherence to the epithelial cell model *in vitro.*
- 6.3.2. To evaluate adherence to mucin *in vitro.*
- 6.3.3. To design an experimental model for the evaluation of *R. inulinivorans* colonization *in vivo.*
- 6.3.4. To determine *R. inulinivorans* colonization in murine model

7. MATERIALS AND METHODS.

7.1. *R. inulinivorans* strains and growth conditions.

R. inulinivorans strain A2-194 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Wild-type (Duncan, 2006), Fla^- mutants Fla^- (this study), spontaneous $rFla^+$ revertants (this study), and pMTL83151-conjugated (WT-C and Fla^--C) strains were routinely grown in an anaerobic chamber (Bactron EZ, Shellab, USA) at 37 °C; the anaerobic condition was generated using a gas mixture containing 87% N_2, 5.4% CO_2, and 7.6% H_2. YCFA-CGM medium was used for the normal growth of *R. inulinivorans* strains. The composition of the medium per liter is as follows: 10 g casitone (BD Difco), 2.5 g yeast extract (BD), 0.9 g sodium chloride (Merck), 0.09 g magnesium sulfate heptahydrate (Merck), 0.09 g calcium chloride (Merck), 0.45 g potassium phosphate dibasic (Merck), 0.45 g potassium phosphate monobasic (Merck), 4 g sodium bicarbonate (Merck), 1 mg resazurin (Sigma-Aldrich), 10 mg haemin (Sigma-Aldrich), 10 µg biotin (Sigma-Aldrich), 10 µg cobalamin (Sigma-Aldrich), 30 µg p-aminobenzoic acid (Sigma-Aldrich), 50 µg folic acid (Sigma-Aldrich), 150 µg pyridoxamine (Sigma-Aldrich), and agar when required (BD Difco). After the medium was autoclaved, cellobiose, glucose, and/or maltose (Merck) were added to a final concentration of 2 mg/mL. Heat-labile vitamins thiamin (Merck) and riboflavin (Sigma-Aldrich) were added to a final concentration of 0.05 µg/mL and the final concentration of short-chain fatty acids (SCFA) were 33 mM acetate (Merck), 9 mM propionate (Sigma-Aldrich) and 1 mM of each valerate, isovalerate, and isobutyrate (Sigma-Aldrich). When required, rifampicin, ciprofloxacin, or thiamphenicol were added to the medium.

7.2. Growth curves.

Overnight cultures of each strain of *R. inulinivorans* growing in YCFA supplemented with cellobiose, glucose, and maltose (YCFA-CGM) broth was used to generate new cultures (inoculum size 2.5% v/v) in fresh YCFA-CGM broth. These cultures were grown in anaerobic conditions until the stationary phase. At 0, 3, 6, 9, 12, and 24 h, an aliquot was serially diluted in reduced PBS and 20 µL of serial dilutions were seeded in YCFA-CGM plates and incubated at 37 °C for 48 h to determine the colony-forming units per mL (CFU/mL). The doubling time (t_d) at 6, 9, and 12 h was calculated with the following formula:

$$Doubling\ time = \frac{Duration \times \ln(2)}{\ln\left(\frac{final\ concentration}{initial\ concentration}\right)}$$

7.3. HT-29 cell line culture conditions

The colorectal adenocarcinoma cell line HT-29 was routinely grown on Dulbecco's Modified Eagle's Medium (Gibco) with 10% of fetal bovine serum (Gibco) (sDMEM) in cell culture flasks (Falcon) at 37 °C in a CO_2 incubator (5% CO_2, ESCO). For all experiments, cells were transferred to 96-wells plates; seeding 50,000 cells per well and incubated for 24h. Next, 96-wells plates were transferred to the anaerobic chamber for adhesion assays 5 h before the assays.

7.4. Minimum inhibitory concentrations to antibiotics.

Minimum inhibitory concentrations (MICs) were determined directly in solid media as previously described (Naganawa et al., 1996). Briefly, rifampicin and thiamphenicol MICs were determined in YCFA-CGM plates. Antibiotics were added to the medium before agarose solidification. Then, YCFA-CGM plates were introduced to the anaerobic chamber 24 h before seeding 50 µL of an overnight culture of *R. inulinivorans*. Once plates with decreasing concentrations of antibiotics were seeded appearance of colonies was determined 72 h after.

7.5. Chemical mutagénesis

R. inulinivorans wild-type strain mutagenesis was performed as previously described elsewhere (Bae et al., 2016). The bacterium was cultured in YCFA-CGM for 18 h at 37 °C; 1 mL was treated with EMS at 2.5, 5, 10, or 20 mg/mL and incubated for 1 h at 37 °C. Next, the EMS-treated bacteria were rinsed three times with PBS and the pellet was resuspended and incubated for 6h in a YCFA-CGM medium. To determine lethality at each concentration of EMS, an aliquot of each overnight culture and EMS-treated culture was serially diluted and 20 µL were seeded in YCFA-CGM plates; after 72 h of incubation CFU/mL were determined. To determine the lower EMS concentration that generates point mutations in the genome of *R. inulinivorans*, an aliquot of the overnight culture and an aliquot of each culture treated with EMS were serially diluted and 20 µL were seeded in YCFA-CGM supplemented with rifampicin at 5 ng/mL (4 times higher than MIC), then, CFU/mL were determined.

7.6. Generation of non-motile mutants and revertant strains in *R. inulinivorans*.

An easy method to look for Fla$^-$ phenotype is by motility analysis in semi-solid media. Then, *R. inulinivorans* Fla$^-$ strains (without flagellum) were generated with EMS at 2.5 mg/mL as previously described. After the incubation on YCFA-CGM broth by 6 h, 10 µL of this suspension were inoculated in the center of semi-solid agar YCFA-CGM plate (agar 0.3%) and incubated for 72 h. Then, bacteria were collected from the inoculation spot and used to inoculate a new semi-solid agar YCFA-CGM plate. This process was repeated 4 times to produce an enrichment of non-motile bacteria. Then, bacteria from the last inoculation spot were recovered and resuspended in 100 µL of PBS, this bacterial suspension was serially diluted, seeded in YCFA-CGM agar plates, and incubated for 48 h. Next, 1000 colonies were picked and inoculated in semi-solid agar YCFA-CGM plates and after 24 h each colony was picked and reinoculated in another plate, this process was repeated 4 times, and every clone that presented minimum motility was discarded. Finally, the 20 colonies with null motility were screened in semi-solid agar YCFA-CGM plates with incubation at 37 °C for 72 h. These bacteria strains were grown in YCFA-CGM broth and stored with 5% DMSO.

To generate spontaneous revertant strains of each Fla$^-$ mutant, consecutive passages were made as previously described elsewhere (Bae et al., 2016). Each Fla$^-$ mutant strain was inoculated in semi-solid agar YCFA-CGM plates and after 72 of incubation the colony was collected and transferred to a new plate, this process was repeated until motility was observed; at that moment from the most external part of the motility-halo, bacteria were recovered to continue with the same procedure until motility was similar to, or higher than, the wild-type strain. These bacteria strains were grown in YCFA-CGM broth and stored with 5% of DMSO.

7.7. *R. inulinivorans* specific primer design and PCR.

Primers were designed in Vector NTI software (version 10.3.0) to amplify the β-fructosidase gene, which is specific for *R. inulinivorans* (Gene ID: 75164096 [LK408_RS17875]). Primers generated (Table 1) were checked in Primer-BLAST to discard amplification in other bacteria or dimer formation. PCRs were made directly from colonies; for this, a portion of each colony was resuspended in 100 µL of molecular biology-grade water (Corning) and incubated at 98° C for 15 min. Then, the bacterial suspensions were centrifuged at 22,000 ×*g* (MC-12, Benchmark), and supernatants were used as templates. Each PCR reaction has 1.25 enzymatic unities of Taq DNA Polymerase, buffer 1×, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, primers (forward and reverse) 25 µM, 1.5 µL of templates, and molecular biology-

grade water to 50 µL. Amplifications were made in a thermocycler (SureCycler 8800, Agilent Technologies) with the following cycling parameters; initial denaturing cycle, 98 °C for 15 min; 35 cycles that include: denaturing at 98 °C for 45 sec, annealing at 55 °C for 45 sec, elongation at 72 °C for 60 sec/kb; final elongation at 72 °C for 5 min. Unspecific primers for bacteria that amplify a fragment of the small ribosomal subunit 16S region of all eubacteria were the same as previously reported by Ramirez-Farias et al., (2008) (Table 1), and the PCR reactions and cycles are the same as mentioned above. PCR reactions were loaded in agarose gel with SYBR™ Safe DNA Gel Stain (Invitrogen) following manufacturer instructions and visualized in a UV transilluminator (MLB-21, MaestroGen).

Table 1. Strain, plasmid, and primers

	Description	Source
***R. inulinivorans* A2-194**	Wild-type strain	Duncan et al. (2006)
***F. prausnitzii* A2-165**	Wild-type strain	Duncan et al. (2006)
***E. coli* CA434**	*E. coli* HB101 with plasmid R702, conjugative strain	D. Williams et al. (1990)
***R. inulinivorans* A2-194 Mutants (Mut)**	19 non-flagellated mutants Mut# (Mut1, Mut2,…, Mut19)	This study
***R. inulinivorans* A2-194 Revertants (Rev)**	7 spontaneous revertants from non-flagellated mutants Rev# (Rev6, Rev8, Rev12, Rev14, Rev15, Rev17, Rev19)	This study
***R. inulinivorans* A2-194/ pMTL83151 (WT-TC)**	Wild-type strain conjugated with plasmid pMTL83151, thiamphenicol-resistant strain	This study
***R. inulinivorans* A2-194 Mut8/pMTL83151 (Mut8-TC)**	Non-flagellated mutant strain conjugated with plasmid pMTL83151, thiamphenicol-resistant strain	This study
***R. inulinivorans* A2-194 Mut12/pMTL83151 (Mut12-TC)**	Non-flagellated mutant strain conjugated with plasmid pMTL83151, thiamphenicol-resistant strain	This study
Plasmid pMTL83151	ColE1 ori; CmR; Pcb102 ori; TarJ. It confers thiamphenicol resistance	J. Heap et al. (2009)
Primer UniF	Sequence (5’ – 3’): GTGGTGCATGGTAGTCGTCA Forward primer for unspecific amplification in Eubacteria (145 – 148 bp amplicon)	Ramirez-Farias et al. (2009)
Primer UniR	Sequence (5’ – 3’): ACGTCGTCCACACCTTCCTC	Ramirez-Farias et al. (2009)

	Reverse primer for unspecific amplification in Eubacteria (145 – 148 bp amplicon)	
Primer Rrec630F	Sequence (5′ – 3′): CGGACTAGAGTGTCGGAGG Forward primer for specific amplification in *Roseburia* genus (266 bp amplicon)	Ramirez-Farias et al. (2009)
Primer Erec870R	Sequence (5′ – 3′): AGTTTCATTCTTGCGAACG Forward primer for specific amplification in *Roseburia* genus (266 bp amplicon)	Ramirez-Farias et al. (2009)
Primer RinuF	Sequence (5′ – 3′): GATACCTATTATATGATCGTTGGCA Forward primer for specific amplification in *R. inulinivorans* (1543 bp amplicon)	This study
Primer RinuF	Sequence (5′ – 3′): ATCAAAGGAAATGACAGCACC Reverse primer for specific amplification in *R. inulinivorans* (1543 bp amplicon)	This study

7.8. Motility and biofilm formation assays.

Motility was evaluated in semi-solid agar YCFA plates (0.3 % agar-agar, BD) supplemented with cellobiose, glucose, and/or maltose (2 mg/mL). Colonies of *R. inulinivorans* were inoculated in the supplemented semi-solid agar YCFA plates with a sterile plastic inoculation needle. After 48 h of incubation, the motility halo was measured on each plate.

Biofilm formation assay was performed as previously described by T. Dapa et al. (2013) with modifications. Overnight cultures of *R. inulinivorans* strains were diluted 1:100 into fresh YCFA-CGM and incubated in TC-treated polystyrene 96-wells plates (Falcon), 300 µL per well, in anaerobic conditions at 37 °C for 24 h. Then, supernatants were carefully removed, wells were gently washed twice with sterile reduced PBS and allowed to dry for 10 min at 37 °C. Next, biofilms were stained with 300 µL of filter-sterilized 0.1% crystal violet (Merck) and incubated at 37 °C for 30 min. The crystal violet was removed from wells, followed by two washes with sterile PBS and the retained dye was extracted with 300 µL of methanol, after incubation for 30 min at room temperature in aerobic conditions, the absorbance at A_{570} was measured with a spectrophotometer (BioTek T800).

7.9. Transmission electron microscopy.

The flagellum lost in Fla^- strains and its recovery in $rFla^+$ strains were determined by transmission electron microscopy (TEM). First, an overnight culture of each strain was used to generate a 5 mL fresh culture in YCFA-CGM broth (5% of overnight culture). These cultures

were incubated at 37 °C for 6 h (exponential phase). Then, 1 mL of each culture was rinsed 3 times with HEPES buffer, previously reduced inside the anaerobic chamber, and finally resuspended in 100 µL of the same buffer. Next, 5 µL of bacterial suspensions were put on Formvar/carbon TEM grids (200 mesh, copper grids, Sigma-Aldrich), incubated for 1 min, and retired with absorbent paper, after 5 µL of uranyl acetate (1% p/v) was added to the grids, incubated for 1 min and retired with absorbent paper. After, the grids were incubated for 5 min at 42 °C and analyzed on a Talos F200C G2 microscope (Thermo Scientific) at 11000× or 92000× magnification.

7.10. Whole genome sequencing and determination of mutations.

R. inulinivorans strains were grown overnight in YCFA-CGM broth. DNA of the strains was extracted using the Wizard Genomic DNA Purification Kit (Promega) following manufacturer instructions, and samples of genomic DNA were sequenced in SeqCenter (Pittsburgh, USA) by Illumina MiSeq system.

For genome assembly and annotation, the Illumina MiSeq raw reads of *R. inulinivorans* DSM 16841 were pre-processed using Trimmomatic v0.36 to trim adaptors and remove low-quality reads (Bolger, Lohse, & Usadel, 2014). Read quality was visualized in FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). *De novo* assembly for Illumina was performed using Velvet v1.2.10 with a quality score > Q30. Contigs obtained were separated by an in-house developed method that includes comparative genomic analyses. The draft genome was annotated using RAST (Rapid Annotation using Subsystem Technology, https://rast.nmpdr.org/rast.cgi) (Brettin et al., 2015)

For the prediction of genetic mutations, the Illumina MiSeq short-read of re-sequencing data from 15 treatments (wild-type and 7 mutant-revertant pairs strains) were pre-processed using Trimmomatic v0.36 to trim adaptors and to remove low-quality reads (Bolger et al., 2014). FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to visualize read quality. To identify mutations, it was used a previous genome assembly as a reference-based alignment. Bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml) was used to map reads implemented on a computational pipeline named breseq. Breseq calls base substitution mutations and small indels by examining the pileup of reads mapped to each position in the reference genome. Biological and relevant mutational events from the evidence are classified as follows: *base substitutions*, *short insertions and deletions*, *large deletions*, *mobile elements insertions*, and *duplications* (Deatherage & Barrick, 2014).

7.11. Adhesion to epithelial cells monolayers.

As previously reported, adhesion to epithelial cells was performed in anaerobic conditions (Bactron EZ, Shellab, USA) as previously reported (McKee et al., 2018). Confluent epithelial cells in a 96-wells plate were transferred to the anaerobic chamber for 4 h before performing the assay and the medium was replaced with reduced DMEM. First, bacteria strains were grown for 6 h in YCFA-CGM, or 4 h in YCFA-CGM plus 2 h in YCFA-CGM with an anti-flagellin antibody (2 µg/mL) (code: ab106146, ABCAM) or its respective isotype control antibody (2 µg/mL) (code: ab93713), rinsed with PBS 3 times and resuspended in reduced DMEM, or reduced DMEM with the respective antibody if necessary. This suspension was considered the input. Therefore serial dilutions were made in PBS and 20 µL of these dilutions were seeded in YCFA-CGM and incubated at 37 °C for 48 h to determine the input CFU/mL. Next, the medium from epithelial cells monolayers was retired and 100 µL of the bacterial suspensions were added. After 1 h of incubation, the monolayers were rinsed 3 times with PBS, 100 µL of PBS were added, epithelial cells were removed from the wells mechanically with pipette tips, and serial dilutions were made. Finally, 20 µL of all serial dilutions were seeded in YCFA-CGM plates and incubated at 37 °C for 48 h to determine the output CFU/mL and the percentage of adhesion.

7.12. Adherence to mucin-agar matrices

Adherence to the mucin-agar layer was performed as previously described (Tsilia, Van den Abbeele, & Van de Wiele, 2015) with modifications. A mucin-agar matrix was generated as follows, a mucin (Sigma-Aldrich) solution (10% p/v in water) and an agar-agar (BD) solution (0.8% p/v in water) were both prepared and autoclaved independently. After these solutions were mixed, a mucin-agar solution (5% p/v – 0.4%) and 500 µL of the resultant solution were added to each well of a 24-wells plate. Bacteria strains were grown for 6 h in YCFA-CGM, or 4 h in YCFA-CGM plus 2 h in YCFA-CGM with an anti-flagellin antibody (code: ab106146, ABCAM) or its respective isotype control antibody (code: 93713, ABCAM), these bacterial suspensions are considered the input, therefore serial dilutions were made in PBS, and 20 µL of these dilutions were seeded in YCFA-CGM and incubated at 37 °C for 48 h to determine the input CFU/mL. Next, 500 µL of the bacterial suspensions were added over each mucin-agar matrix. After 1 h of incubation the monolayers were rinsed 3 times with 1 mL of PBS, and 1000 µL of PBS were added. The matrices containing the adhered bacteria were

ground mechanically with pipette tips, and serial dilutions of the final suspension were made. Finally, all serial dilutions were seeded in YCFA-CGM plates and incubated at 37 °C for 48 h to determine the output CFU/mL and the percentage of adhesion to the mucin-agar layer.

7.13. Mice housing

Conventionally-raised C57BL/6 mice (8 – 12 weeks old) were obtained from the breeding colony at Facultad de Ciencias de la Vida, Universidad Andrés Bello, derived from Jackson Laboratories. Mice were maintained with 12 h cycle of light and darkness at 20 – 25°C with 40–60% humidity. Mice have ad libitum access to food RMH 3000 (Prolab, USA) and autoclaved water. Paper pellets for bedding and cages were autoclaved before use. All the procedures were made in a changing station (model cs5 evo, Tecniplast).

7.14. Intestinal microbiota reduction

In order to reduce the colonization resistance of conventionally-raised mice (N=3, 1 male and 2 female), an antibiotic cocktail containing vancomycin (20 mg/mL), kanamycin (40 mg/mL), ampicillin (40 mg/mL), and metronidazole (10 mg/mL) (VKAM) was used to treat animals for 14 consecutive days. The dose of every antibiotic per kg of animal was as follows, vancomycin (50 mg/kg), kanamycin (100 mg/kg), ampicillin (100 mg/kg), and metronidazole (25 mg/kg). Feces from mice (30 – 50 mg) were collected every 7 days, resuspended in 500 µL of sterile PBS, and vortexed for 3 min until feces were grounded. Immediately, an aliquot was transferred to the anaerobic chamber, serially diluted in reduced PBS, and 20 µL were seeded on YCFA-CGM plates. Finally, after 72 h of incubation, the CFU/g of feces was determined for each mouse.

7.15. Thiamphenicol resistant strains

Thiamphenicol-resistant strains were obtained through conjugation with pMTL83151 plasmid in WT and Fla- mutant strains as previously reported elsewhere with modifications (Sheridan 2019). *Escherichia coli* (*E. coli*) CA434/pMTL83151 strain was grown in 20 mL of Luria-Bertani (LB) overnight in aerobic conditions, this culture was centrifuged at 3871 ×*g* for 10 min and rinsed with PBS, this process was repeated 3 times and the pellet was introduced to the anaerobic chamber. The *E. coli CA*434 pellet was resuspended with 500 µL of an overnight culture of *R. inulinivorans* WT or Fla- strains and 100 µL from this *E. coli* CA434 –

R. inulinivorans suspensions were spotted on YCFA-CGM plates (10 µL each spot). After 48 h of incubation, spots were collected with 1 mL of reduced PBS and 100 µL were seeded in YCFA-CGM plates supplemented with thiamphenicol (0.01 mg/mL, for selection of plasmid resistance) and ciprofloxacin (0.02 mg/mL, for counterselection of *E. coli CA*434), and incubated for 48 h in anaerobic conditions. Ciprofloxacin-Thiamphenicol resistant colonies were seeded individually in a new YCFA-CGM-TC plate, and a PCR from colonies was made using *R. inulinivorans* A2-194 specific primers (Table 1) to confirm that isolated colonies were *R. inulinivorans*.

7.16. Intestinal colonization assay.

Because the microbiota from feces could be restored after the treatment with antibiotics, the conjugated thiamphenicol-resistant strains WT-TC, Mut8-TC, and Mut12-TC were used. For this purpose, the MIC in plates for the *R. inulinivorans* strains and mice feces (N=3) were determined, as previously described. Then, a higher concentration than necessary to avoid bacterial growth from feces in plates after 72 h of incubation was selected to continue with the experiments.

Conventionally-raised mice (male and female) were treated with a VKAM cocktail for 7 days. After 2 resting days without antibiotic treatment mice were inoculated with sterile YCFA-CGM (control group, N=3), 10^8 CFU of *R. inulinivorans* WT-TC strain (N=5), or two Fla$^-$-TC strains (N=5 for each mutant), for 3 consecutive days to determine the presence of thiamphenicol-resistant bacteria in feces. The stool samples were collected the day before inoculation and 5 days after the last inoculation. Feces from mice (30 – 50 mg) were collected in 1.5 mL microtubes, resuspended in 500 µL of sterile PBS, and vortexed for 3 min until feces were grounded. Immediately, the suspensions were transferred to the anaerobic chamber, serially diluted in reduced PBS, and 20 µL were seeded on YCFA-CGM supplemented with thiamphenicol at 0.1 mg/mL under anaerobic conditions. Finally, after 72 h of incubation, the CFU/mg of feces was determined for each mouse.

7.17. Statistical analysis.

For statistical analysis, GraphPad Prism 7.0 was used. To determine whether data were parametric or no parametric for each experiment, normality was determined by the Shapiro-Wilk test, and homoscedasticity was determined by the Brown-Forsythe test.

For analysis of differences between strains or conditions on each experiment, a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test as *post hoc* was performed in parametric data. In contrast, a Kruskal-Wallis test with Dunn's multiple comparison test as *post hoc* was performed for no parametric data. Analysis performed for each experiment is expressed on each figure legend.

8. RESULTS

8.1. Random mutagenesis in *R. inulinivorans* with EMS

R. inulinivorans is a strictly anaerobic, slightly curve bacillus showing subterminal flagella. This specie belongs to a genus without site-directed mutagenesis tools as many Firmicutes-phylum members. However, random mutagenesis by chemical compounds, such as EMS, is a valuable tool that allows genetic studies in this kind of bacteria (Bae et al., 2016). In order to determine the flagellum relevance in this specie, it was selected EMS treatment for chemical mutagenesis to obtain non-flagellated strains by punctual mutations in *R. inulinivorans* chromosome. Punctual mutations are preferred over deletions due are easier to revert by spontaneous mutations, simplifying the revertant generation after.

To determine the best EMS concentration to generate mutations in *R. inulinivorans*, lethality and generation of rifampicin-resistant bacteria were determined at 4 different EMS concentrations, 2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL, the same range of concentration used for another Firmicutes belonging bacteria by Bae et al., (2016). It was selected one time of exposition to EMS since both factors concentration and exposition time affect in the same way the number of mutations per cell.

EMS generates random single-base mutations in the genome, then, it can produce non-viable phenotypes or decrease the fitness of the mutants. Both could be observed as a survival decrease in cultures exposed to this mutagen. Figure 2A shows that *R. inulinivorans* survival decreases inversely to EMS concentration, from 30% survival at 2.5 mg/mL to 95% at 20 mg/mL. Another strategy to evaluate the mutagenic effect of EMS is rifampicin resistance because this phenotype is the result of a single base mutation, then, it can be used as a marker of the effectiveness of EMS treatment as a mutagen. Therefore, the concentration of rifampicin in solid media was first determined to avoid the growth of wild-type *R. inulinivorans.* The growth is inhibited from 1.25 ng/mL in YCFA-CGM plates (Table 2), then a concentration of 10 ng/mL was used for determining the generation of rifampicin-resistant strains. It can be observed that the percentage of rifampicin-resistant mutants in the culture increases concomitantly with the EMS concentration (Figure 2B). No rifampicin-resistant mutants were detected without the EMS treatment or concentrations equal to or less than 1.25 mg/mL (data not shown), while from 2.5 mg/mL to 20 mg/mL, the percentage of this type of mutants in the culture increased from 0.005% to 0.02%, respectively. For the following aim, generating the minimum number of mutations in our culture is necessary, so we have selected the 2.5 mg/mL EMS concentration to produce non-flagellated mutants due it was the lower

concentration of EMS tested which generates rifampicin-resistant strains and also shown the higher survival.

Table 2. Solid-medium growth at increasing rifampicin concentrations in the wild-type strain.

	0.16 ng/mL	0.31 ng/mL	0.63 ng/mL	1.25 ng/mL	2.5 ng/mL	5 ng/mL	10 ng/mL
R. inulinivorans.	+	+	+	-	-	-	-

* No growth, -; Growth, +.

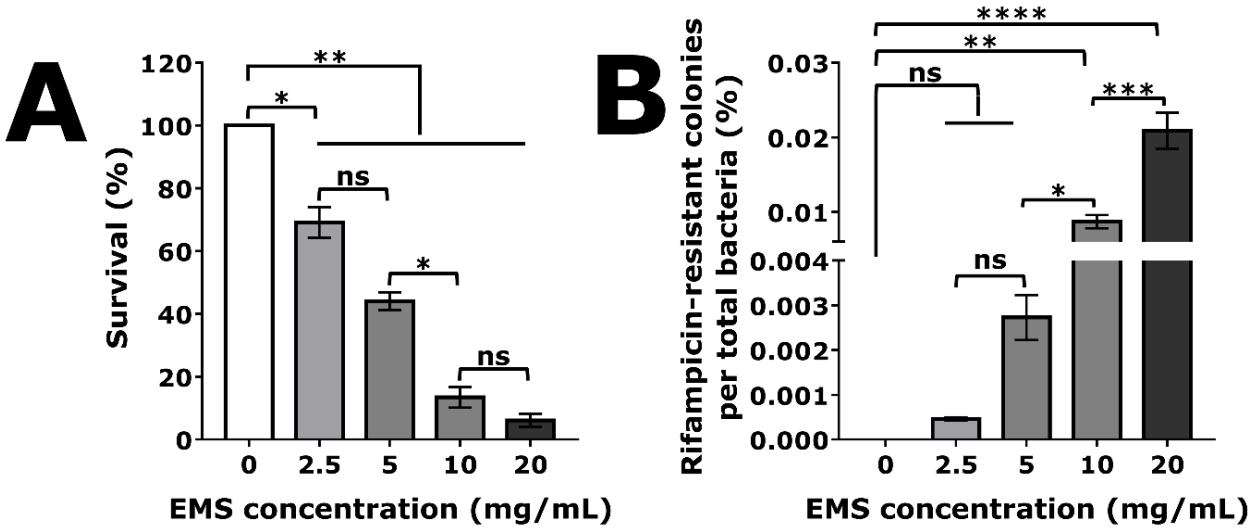


Figure 2. EMS concentration dependence in survival and generation of rifampicin-resistant mutants in *R. inulinivorans*. Overnight cultures of *R. inulinivorans* grown in YCFA-CGM medium were treated for 1 h with EMS at 2.5, 5, 10, or 20 mg/mL. (A) Survival percentage relative to control was calculated based on CFU/mL obtained on YCFA-CGM plates at each concentration immediately after treatment. (B) Rifampicin-resistant CFU grown in YCFA-CGM supplemented with 5 ng/mL rifampicin were used to calculate the percentage of rifampicin-resistant colonies per total bacteria after the treatment with EMS and 6 h of recovery for each concentration (N=3, ANOVA with Tukey's multiple comparison test as post hoc; ns, no significative; $p<0.05$, *; $p<0.005$, **; $p<0.001$, ***; $p<0.0001$, ****; error: SEM).

8.2. Generation of no-motile and revertant strains in *R. inulinivorans*.

To generate non-flagellated strains of *R. inulinivorans* it was first evaluated the flagellar motility in semi-solid YCFA plates without sugar or supplemented with cellobiose, glucose, and/or maltose because it has been reported that motility in the *Roseburia* genus

could be influenced by sugar present in the medium (Sheridan, 2014). When compared, in YCFA media, sugar supplementation is required for motility, but no differences exist between supplementation with glucose, maltose, or cellobiose, nor when three sugars were used together after 48 h of incubation (Figure 3). Therefore, although no differences were observed, we decided to use YCFA-CGM for subsequent experiments because it was the routinely medium for growing *R. inulinivorans*.

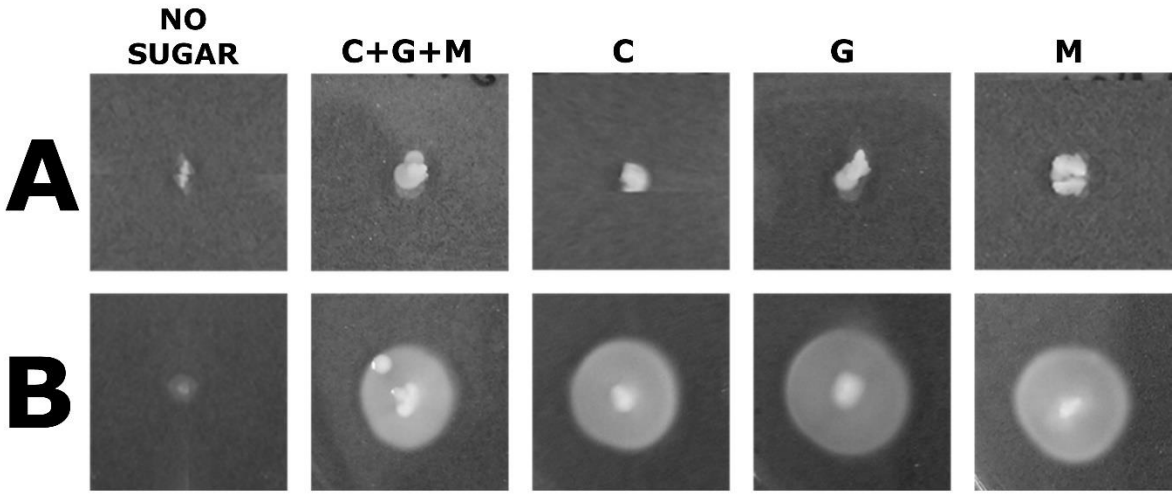


Figure 3. Motility in YCFA plates in presence of different sugars. (A) *F. prausnitzii* (no motility control) or (B) *R. inulinivorans* colonies growing on YCFA-CGM plates were used to inoculate the center of semi-solid YCFA plates without sugar supplementation, supplemented with cellobiose, 2mg/mL (C); glucose, 2 mg/mL (G); maltose, 2 mg/mL (M); or the three sugars (C+G+M), 2 mg/mL each. After 48 h incubation motility halos were observed. Representative figure (N=3).

Using the concentration previously determined of EMS (2.5 mg/mL) a random mutagenesis was performed on *R. inulinivorans* to generate non-flagellated strains. After four passages in semi-solid YCFA-CGM plates to increase the number of no-motile mutants, bacteria were collected from the center of semi-solid YCFA-CGM plates and seeded in a YCFA-CGM plate. Thus, 1000 colonies from these plates were inoculated in semi-solid plates and after four passages 19 colonies maintained the total loss of motility which were evaluated to belong *Roseburia* genus.

All the colonies had slightly curved rod morphology when observed by phase contrast microscopy, as the wild-type strain (Figure 4A), and *Roseburia* genus belonging were confirmed for all the strains by PCR using specific primers (Figure 4B) previously described by Ramirez-Farias et al., (2009). Then all the strains are considered no-motile *R. inulinivorans*

mutants (identified as Mut1, Mut2, …, Mut19). These mutants also had the same colonial morphology characteristics after 72 h incubation, except for the size of mutants Mut4, Mut9, Mut13, and Mut18, which were smaller than wild-type (data not shown). As random chemical mutagenesis could generate more than one mutation per cell, these mutations may decrease the fitness of mutants. To evaluate this, growth curves were performed in YCFA-CGM broth. Most of the mutants have similar growth to the wild-type *R. inulinivorans*, except mutants Mut4, Mut9, and Mut13, which had a more extensive lag phase than others but at 24 h of incubation, Mut4 and Mut9 have a similar CFU/mL, while Mut13 has a lower CFU/mL at the same time (Figure 5). Based on these results, it was decided to take off these 4 mutants (Mut4, Mut9, Mut13, and Mut18) to generate revertant strains.

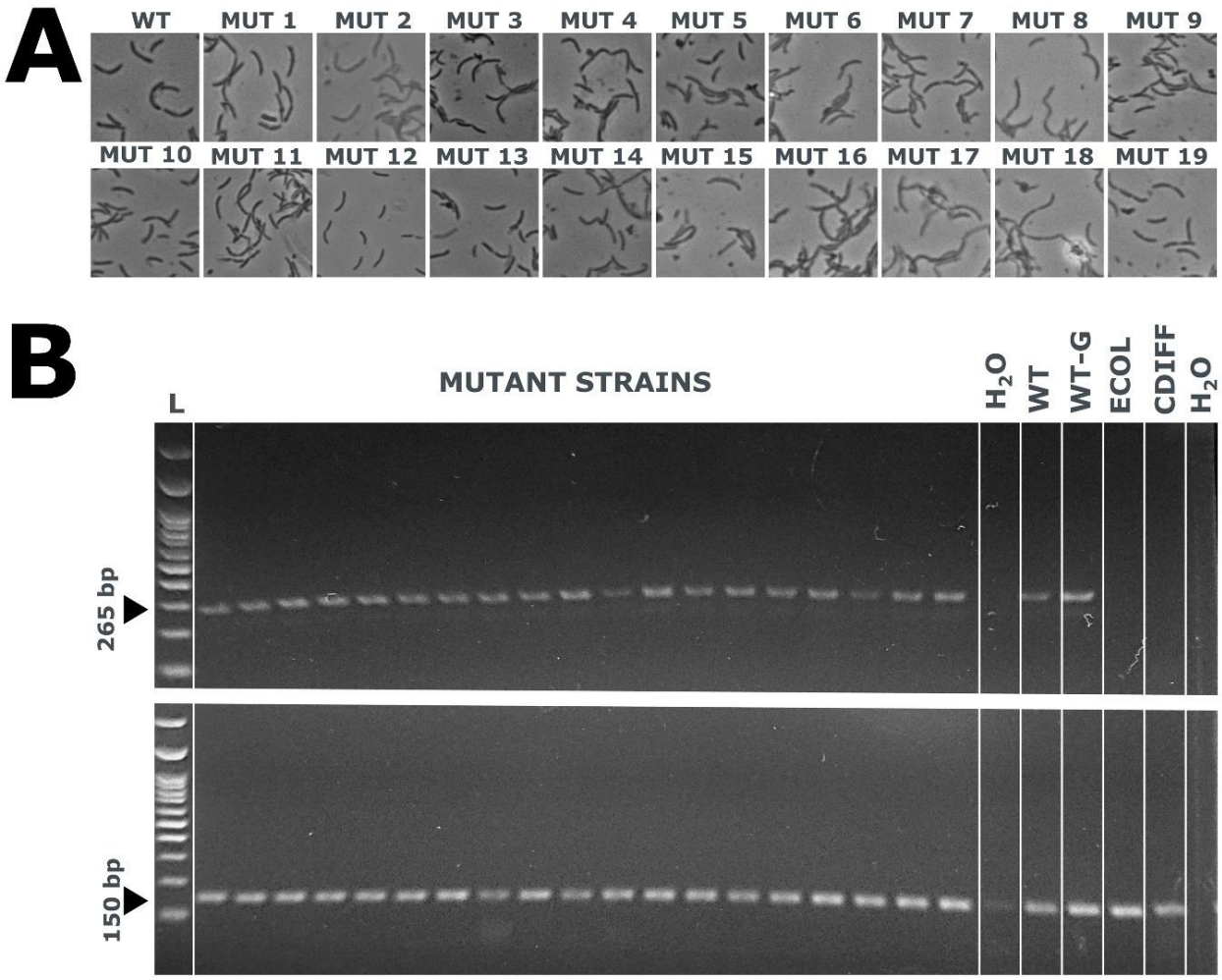


Figure 4. Genus confirmation for no motile mutants. Nineteen no-motile strains obtained after mutagenesis were tested by phase contrast microscopy (A), and PCR (B). (A) overnight cultures of each mutant (MUT1 to MUT19) and the wild-type (WT) strains were diluted 1:10 in reduced PBS and observed in a phase contrast microscopy at 1000× magnification. (B) colonies of each strain were collected to perform colony PCRs using bacterial unspecific primers (bottom) and *Roseburia* genus-specific primers (upper), then PCRs results were observed by DNA-gel electrophoresis. As positive PCR controls for the genus, wild-type strain (WT) and its DNA extracted by the phenol-chloroform method (WT-G) were used. For negative PCR controls, *E. coli* (ECOL) and *C. difficile* (CDIFF) were used.

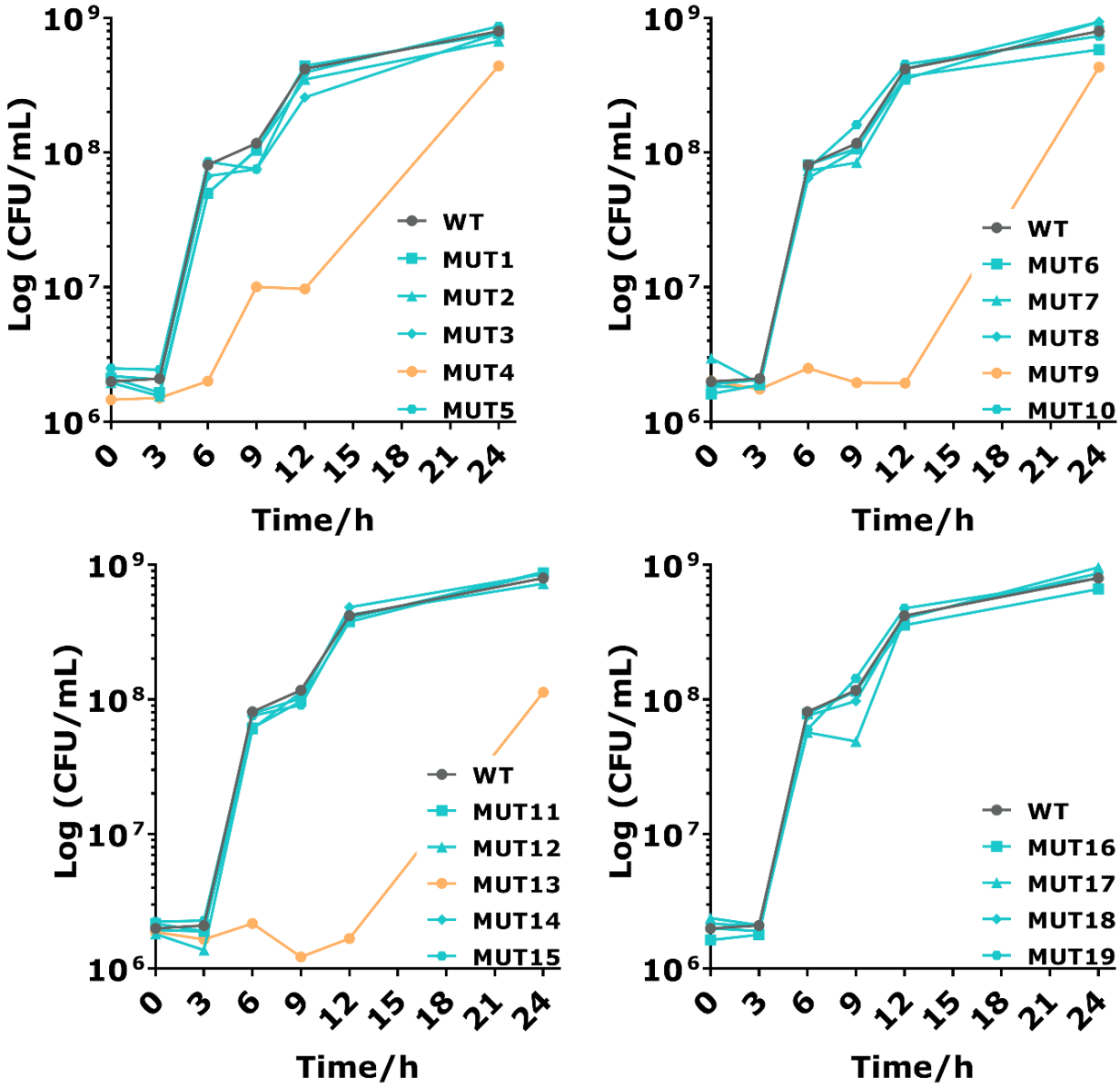


Figure 5. Mutant growth curves. Growth curves were made for the wild-type and the nineteen no-motile mutant strains. First, YCFA-CGM broths were inoculated with overnight cultures of each mutant strain (inoculum size = 2.5% v/v), then an aliquot was taken, serially diluted, and seeded on YCFA-CGM plates at 0, 3, 6, 9, 12, and 24 h of incubation. After 48 h, colonies in plates were counted and CFU/mL was calculated for each time point and strain. Similar growth curves to the wild-type are colored turquoise, while no similar are colored orange. (Representative figure of N=3).

8.3. Generation of motility revertants by spontaneous mutagenesis.

In bacteria, spontaneous mutations occur during DNA replication at a rate of 10^{-10} to 10^{-9} per nucleotide per generation without strong selective pressure and could be influenced by growth conditions (Jeremy W. Schroeder et al.). Then, multiple passages in semi-solid plates were made to generate spontaneous revertants for no motile mutants. Each mutant was inoculated in semi-solid YCFA-CGM plates and 72 h after from the border of the inoculum bacteria were collected and reinoculated in a new semi-solid YCFA-CGM plate. This process was repeated for 50 to 60 generations when appears the revertants for mutants Mut6, Mut8, Mut12, Mut14, Mut15, Mut17, and Mut19. Reversion for the other mutants was not generated even after 80 generations. Revertant names are assigned by mutants [Rev(mutant number), *e. g.*, for Mut6, revertant is Rev6)]. Thus, 7 strain groups (mutant-revertant) were obtained for the following characterization.

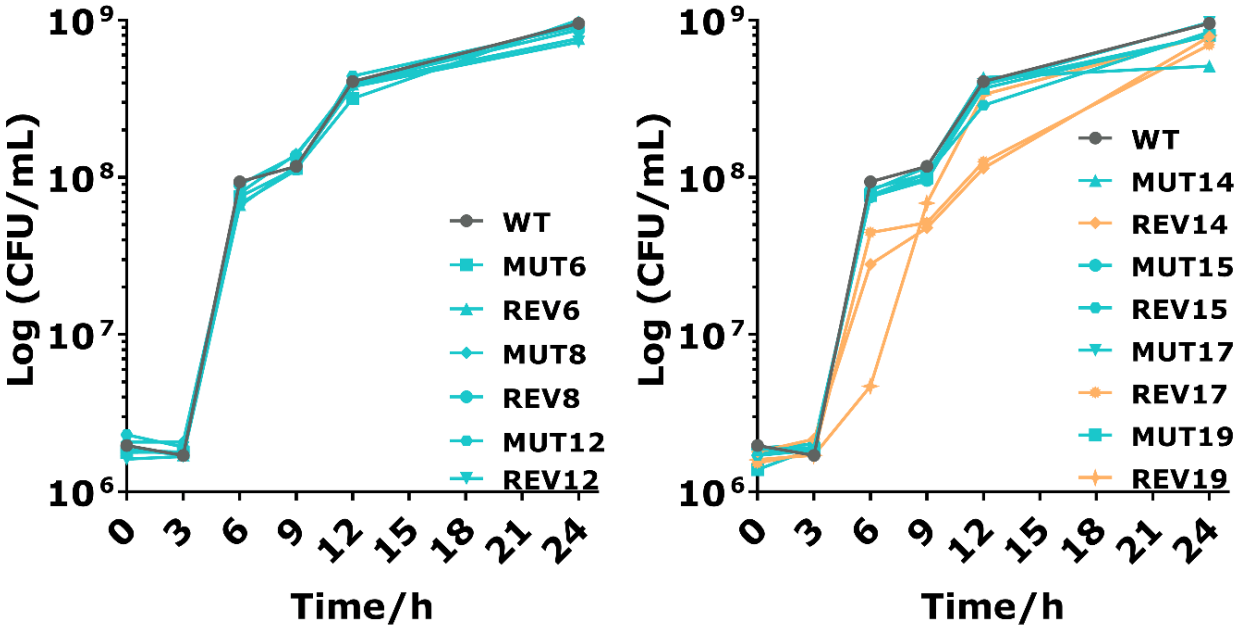


Figure 6. Mutants and spontaneous revertants growth curves. Growth curves were made for the wild-type and 7 pairs of mutant-revertant strains (inoculum size = 2.5% v/v), then an aliquot was taken, serially diluted, and seeded on YCFA-CGM plates at 0, 3, 6, 9, 12, and 24 h of incubation. After 48 h, colonies in plates were counted and CFU/mL was calculated for each time point and strain. Similar growth curves to the wild-type are colored turquoise, while no similar are colored orange. Representative figure of N=3.

8.4. Phenotype characterization of non-motile mutants and revertant strains.

Phenotypical changes can occur when strains pass through multiple generations(Lind & Andersson, 2008). Thus, the growth of revertant strains was compared with mutants and wild-type strains in YCFA-CGM broth. It was observed that revertants Rev6, Rev8, Rev12, and Rev15 have similar growth curves to the mutants and wild-type strains, while Rev14, Rev17, and Rev19 have slightly different growth curves (Figure 6), and the same was observed in doubling time (t_d) at 6, 9, or 12 h (Table 3), these differences were no significative. Then, motility was determined for each strain group (S[number of mutant-revertant]) in semi-solid YCFA plates at 72 h of incubation. Figure 7 shows that revertants have higher motility than wild type. Rev14, Rev15, and Rev17 have a double diameter in motility halo, while Rev6, Rev8, Rev12, and Rev19 have 5-, 3-, 4-, and 3-times higher motility than the wild type, respectively. These results confirm the total loss of swimming motility in mutant strains and suggest that revertants present more than one mutation that affects motility in *R. inulinivorans*.

Tabla 3. Doubling time at 6, 9, and 12 h for wild-type, mutant, and revertant strains.

Strain	t_d at 6 h		t_d at 9 h		t_d at 12 h	
	t_d	SD	t_d	SD	t_d	SD
Wild type	1.04	0.01	1.47	0.01	1.52	0.03
Mut6	1.09	0.04	1.49	0.11	1.58	0.05
Rev6	1.13	0.07	1.47	0.03	1.52	0.07
Mut8	1.13	0.04	1.47	0.07	1.58	0.02
Rev8	1.03	0.01	1.40	0.03	1.51	0.03
Mut12	1.17	0.02	1.54	0.03	1.53	0.01
Rev12	1.12	0.06	1.45	0.12	1.52	0.10
Mut14	1.09	0.08	1.66	0.22	1.51	0.06
Rev14	1.52	0.09	1.91	0.09	2.00	0.02
Mut15	1.13	0.02	1.59	0.01	1.56	0.07
Rev15	1.06	0.01	1.51	0.01	1.62	0.01
Mut17	1.10	0.01	1.53	0.03	1.54	0.01
Rev17	1.23	0.03	1.78	0.07	1.89	0.09
Mut19	1.05	0.13	1.46	0.09	1.49	0.11
Rev19	3.86	0.31	1.66	0.01	1.55	0.02

* Turquoise color remark strains with growth differences observed in Figure 5.

No motile phenotype is the product of two phenomena, flagellum loss or a non-functional flagellum which is present in bacteria but is paralyzed (Smith & Lefebvre, 1997). In order to determine which kind of mutants were our non-motile mutants, each strain was observed by TEM. In bacteria, flagellum expression occurs in all the phases of the growth curve but mainly during the log phase (Chilcott & Hughes, 2000). Therefore, all the strains

were cultured in YCFA-CGM broth for 6 h (until the log phase). At that time, cells were washed with HEPES buffer and loaded in Formvar/Carbon grids. When observed at 11000× magnification, wild-type and revertant strains present multiple flagella surrounding bacteria, conversely, in no-motile mutant strains flagella were not detected (Figure 8). These results confirm that all the no-motile mutants evaluated are non-flagellated *R. inulinivorans* strains.

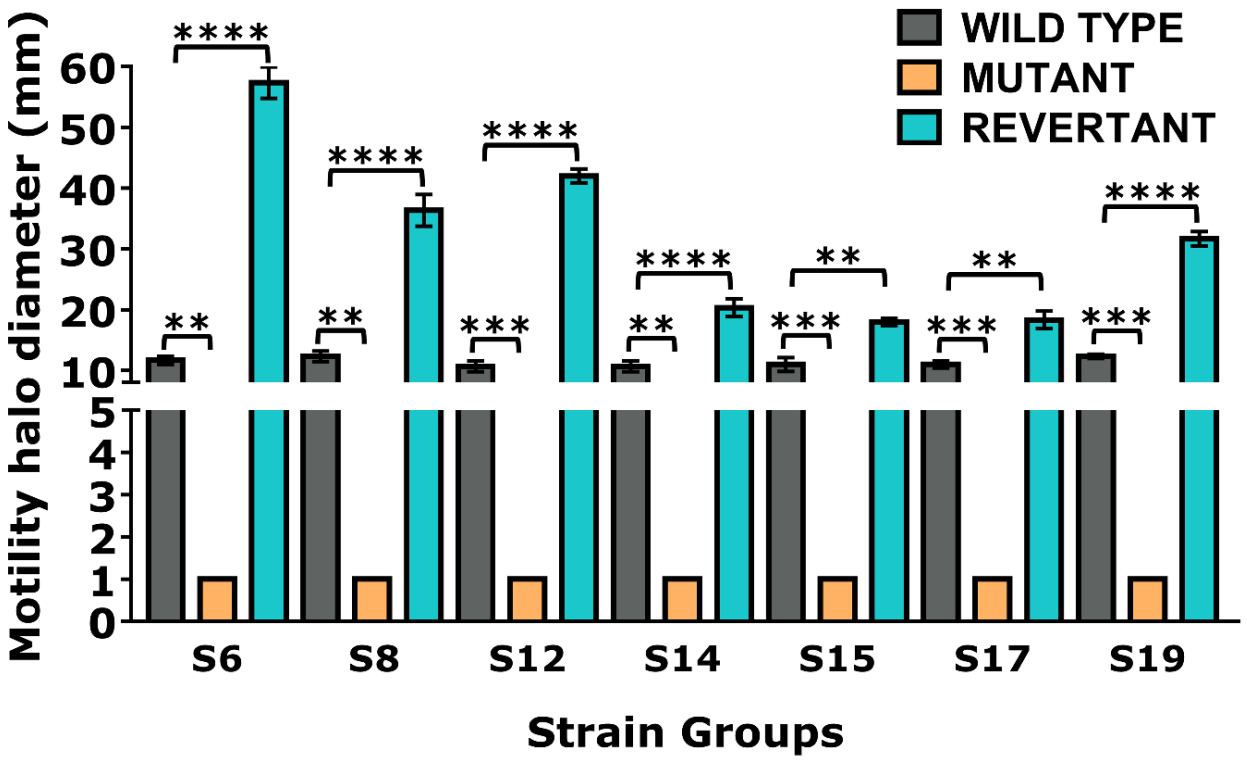


Figure 7. Motility of wild type, mutants, and revertants strains of *R. inulinivorans*. The motility for all the strains was determined in semi-solid YCFA-CGM plates. A colony of each strain growing on YCFA-CGM plates was used to inoculate semi-solid YCFA-CGM plates with sterile plastic needles. After 72 h incubation motility halos were measured (N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; $p<0.005$, **; $p<0.001$, ***; $p<0.0001$, ****; error: SEM).

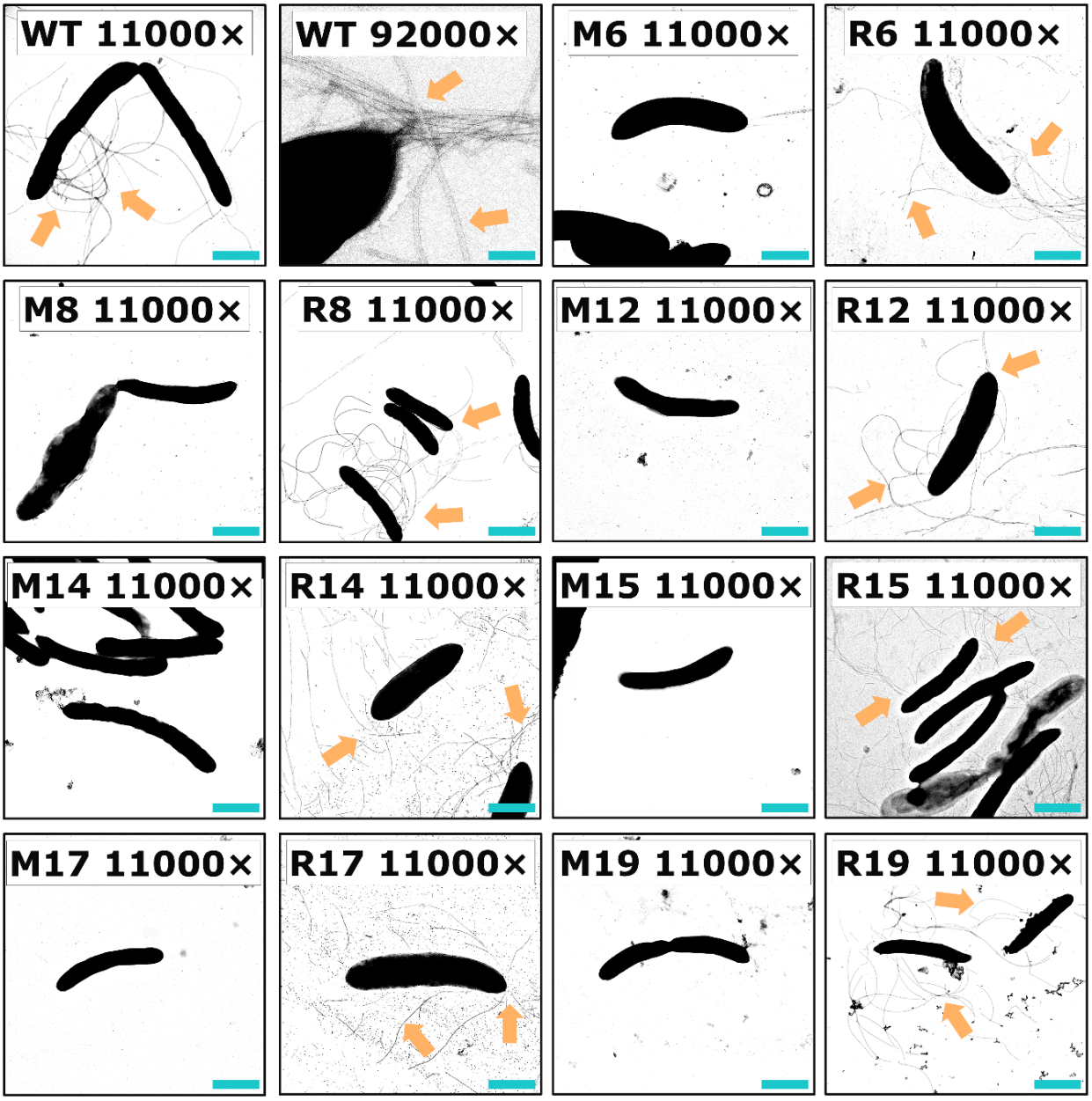


Figure 8. Flagellum detection by TEM. Strains were grown for 6 h in YCFA-CGM broth after an aliquot was rinsed 3 times with reduced HEPES buffer. Next, samples were resuspended in HEPES buffer, 5 µL were loaded on Formvar/carbon grids and uranyl acetate (1% p/v) was used as negative staining. Finally, all samples were observed on a Talos F200C G2 microscope at 11000× (Barr, 1 µm) and 92000× (Wild type; Barr 100 nm). Orange arrows show flagellum in the image.

8.5. Genetic analysis of non-flagellated phenotype

Whole genome sequencing (WGS) is a powerful tool that provides valuable genetic information about strains. Thus, when unknown mutations have occurred WGS allows elucidation of which genes have been mutated. Many gene mutations could be involved in the non-flagellated phenotype, such as mutations in transcriptional regulators or flagellum-structural genes (Bae et al., 2016). Thus, mutant and revertant strains were sequenced and compared to the wild-type genome to determine the mutations generated in flagellar-related genes. There were observed from 12 to 19 mutations per cell in the EMS-mutagenized mutant strains. Then, the relevant genes associated to the flagellar machinery were analyzed. Mut6, Mut8, and Mut14 have the same nonsense mutation in the *fliP* gene which codes for a protein of 282 aa from the flagellum-export apparatus (Figure 1), where the mutation generates a stop codon (CAG→TAG, Q126*) (Figure 9A); the same gene was mutated in Mut19 and also generates a nonsense mutation but in another amino acid of the protein (TGG→TGA, W270*) (Table 4). Another gene mutated was *fliM* which codes for a 326 aa protein part of the C-ring of the flagellum (Figure 1). This mutation was only detected in Mut12 (TGG→TGA, W167*) (Figure 10A). Mut15 and Mut17 have a mutation in the *flhB* gene which codes for a 377 aa protein that as *fliP,* codes for a protein of the flagellum-export apparatus (TGG→TGA, W17*) (Table 4). Thus, all mutations in flagellar machinery determined in non-flagellate strains were nonsense mutations that explain the phenotype.

When revertants strains mutations were assessed, true reversions and pseudo-reversions were observed, while in some revertants no concordant genetic reversions were found in the flagellar machinery. Rev14, Rev15, and Rev17 strains conserved the nonsense mutation from their respective mutant and presented no new mutations in flagellum-related genes (Table 4). Rev12 has a true reversion in *fliM* mutation (TGA→TGG, *167W) but has a new single base change in this gene which produce a missense mutation (CGT→TGT, R42C) (Figure 10A), and there were found a new nonsense mutation in the *fliF* gene which code for the protein that forms the MS-ring of the flagellum (TGG→TGA, W528*) (Figure 10B). Rev19 has changed the nonsense mutation by a missense mutation (TAG→TGC, *270C) in *fliP* and no more mutations in flagellar machinery were detected in this strain (Table 3). Finally, revertants of Mut6 and Mut8 have reverted the mutation in different ways by pseudo-reversions, Rev6 has a single base insertion in the stop codon of the mutant (TAG →TTA-G, *126L) which produces a frameshift that generates changes from this amino acid, including a stop codon that generates a shorter protein (155 aa) than wild-type FliP (282 aa). Reversion in Rev8 occurs by the change of the nonsense mutation for a missense mutation compared to

the wild-type sequence (TAG →CAG, *126Y) (Figure 9A). Rev6 and Rev8 have missense mutations in the chemotaxis protein CheY, a protein of 119 amino acids, and these mutations occur in different amino acids from the aminoacidic sequence for Rev6 (GAC→GAA, D99E) and Rev8 (GCA→GAA, A113E) (Figure 9B). Based on these results, only strain groups S6, S8, S12, and S19 present mutations and reversions concordant with the phenotypes observed. Also, these results confirm that *fliP* and *fliM* are functional and necessary for flagellum presence in *R. inulinivorans.*

Based on these results, only S6, S8, and S12 were selected for the following experiments because their phenotypes correlated with their mutations and were confirmed by the reversion mutations in revertant strains. In contrast, S19, although genetically consistent, the Rev19 revertant presents an impaired growth that could hinder later interpretations.

Table 4 Flagellar mutations in *R. inulinivorans* strains.

Gene	Description of flagellar protein	Amino acid mutation (Base changed) [Total amino acids]	Strains
cheY	Chemotaxis regulator of the flagellar motor	D99E (GAC→GAA) [119 aa]	Rev6
cheY	Chemotaxis regulator of the flagellar motor	A113E (GCA→GAA) [119 aa]	Rev8
flhB	Flagellar biosynthesis protein	W17* (TGG→TGA) [377 aa]	Mut15, Rev15, Mut17, Rev17
Intergenic before *fliD*	Flagellar hook-associated protein	Intergenic (G→A) 78 bp before *fliD* gene	Mut19, Rev19
fliF	Flagellar M-ring protein	W528* (TGG→TGA) [534 aa]	Rev12
fliM	Flagellar motor switch protein	W167* (TGG→TGA) [326 aa]	Mut12
fliM	Flagellar motor switch protein	R42C (CGT→TGT) [326 aa]	Rev12
fliP	Flagellar biosynthesis protein	Q126* (CAG→TAG) [282 aa]	Mut6, Mut8, Mut14, Rev14
fliP	Flagellar biosynthesis protein	Q126L (CAG→TTA G) [282 aa]	Rev6
fliP	Flagellar biosynthesis protein	Q126Y (CAG→TAT) [282 aa]	Rev8
fliP	Flagellar biosynthesis protein	W270* (TGG→TGA) [282 aa]	Mut19
fliP	Flagellar biosynthesis protein	W270C (TGG→TGC) [282 aa]	Rev19

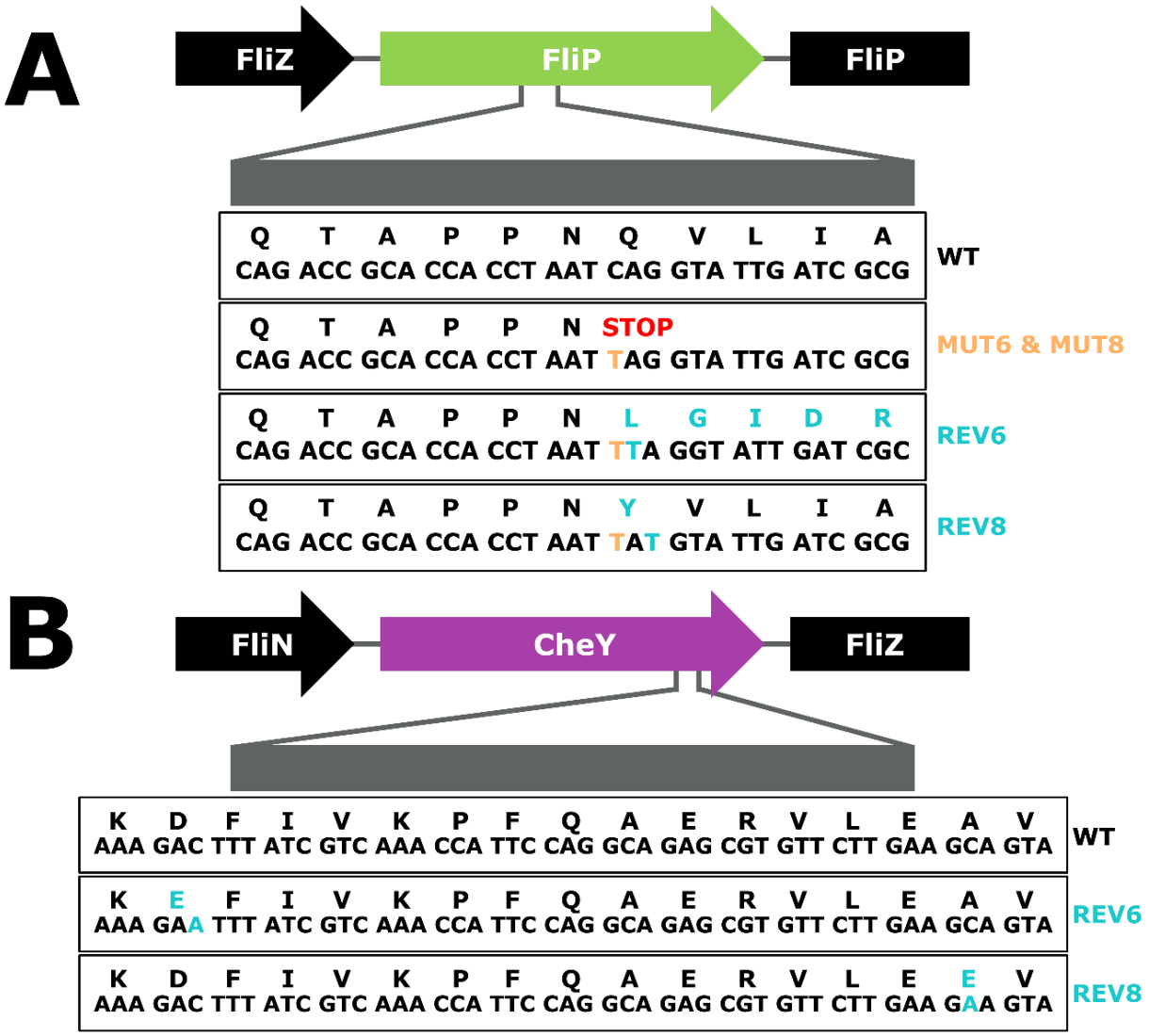


Figure 9. Flagellar-machinery mutations in Mut6, Rev6, Mut8 and Rev8. Wild type, Mut6, Rev6, Mut8, and Rev8 strains were sequenced in Illumina MiSeq. Raw reads were assembled using Velvet v1.2.10 (Quality score >Q30) and annotated using RAST. The prediction of genetic mutations in mutants and revertants was performed by mapping in Bowtie2 with the Breseq pipeline. Mutations that affect flagellar-machinery genes are colored orange (found initially in mutant strains) and turquoise (found in revertant strains). Changes in aminoacidic sequence are shown over nucleotide sequences. Mutations on FliP occur in amino acid 126 for all the stains (282 aa protein); mutations on CheY occur in amino acid 99 and 113 (119 aa protein), in Rev6 and Rev8 strains, respectively.

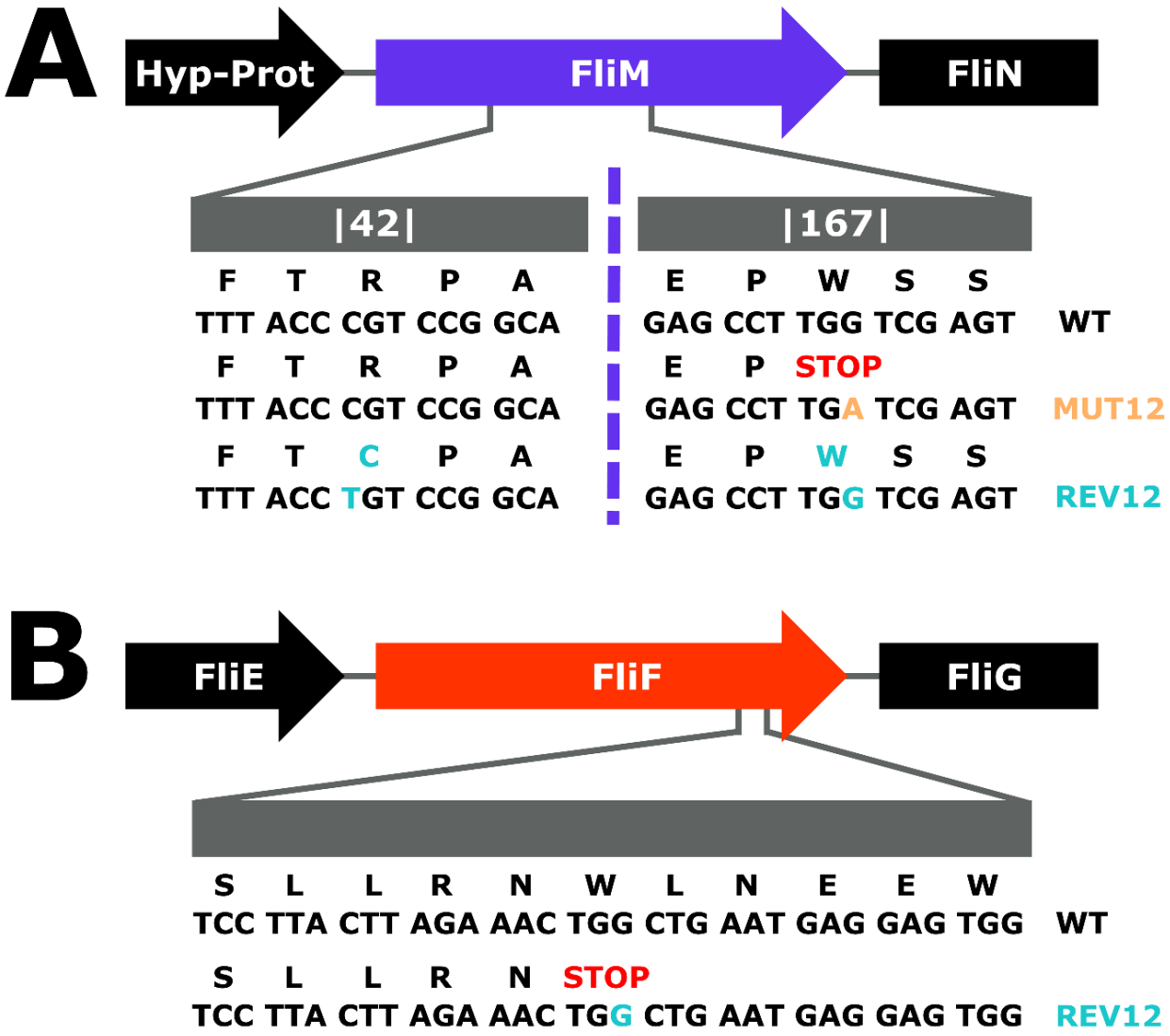


Figure 10. Flagellar-machinery mutations in Mut12 and Rev12. Wild type, Mut12, and Rev12 strains were sequenced in Illumina MiSeq. Raw reads were assembled using Velvet v1.2.10 (Quality score >Q30) and annotated using RAST. The prediction of genetic mutations in mutants and revertants was performed by mapping in Bowtie2 with the Breseq pipeline. Mutations that affect flagellar-machinery genes are colored orange (found initially in mutant strains) and turquoise (found in revertant strains). Changes in aminoacidic sequence are shown over nucleotide sequences. Mutations on FliM occur in amino acids 167 and 42 for mutant and revertant (326 aa protein) in Mut12 and Rev12, respectively; mutation on FliF occurs in amino acid 528 (534 aa protein) in Rev12.

8.6. Flagellum relevance in adherence to epithelial cells.

An essential step for colonizing niches by bacteria is adherence to that niche. Thus bacteria have developed different mechanisms to adhere to many surfaces utilizing adhesins (Juge, 2012). In the gut, the epithelial barrier of the mucosa is one of the principal components that bacteria can recognize and adhere to through adhesins, such as flagellum. Therefore, adherence to epithelial cells was evaluated in the HT-29 cell line monolayers in our selected non-flagellated mutants and revertants. For this, *R. inulinivorans* strains Mut6, Rev6, Mut8, Rev8, Mut12, Rev12, and wild type were grown in YCFA-CGM broth for 6 h (log phase). At this time bacteria were rinsed with PBS and resuspended in previously reduced DMEM medium (input bacteria), then cell suspensions were seeded over confluent cell monolayers growing in 96-well plates and incubated for 1 h under anaerobic conditions. After incubation, each well was gently rinsed with PBS 3 times, and adhered bacteria were recovered in PBS after mechanical disruption of the cell monolayer (output bacteria). Compared to the wild-type, the three non-flagellated strains showed an increased adherence to the epithelial cells, while revertant strains are less adherent than the wild-type to HT-29 epithelial cells (Figure 11C). It has been described elsewhere that motility can be inhibited, and flagellar genes are under-expressed when bacteria are exposed to anti-flagellin antibodies in a non-species-specific way (Cullender et al., 2013). Hence, adhesion to epithelial cells was evaluated in the wild-type strain in the presence of anti-flagellin antibodies. Bacteria were grown for 4 h in YCFA-CGM broth and the culture was divided into three microtubes and anti-flagellin antibody, isotype-control antibody, or PBS was added to each microtube and were incubated for 2 h more. Then, each culture was rinsed and resuspended in reduced DMEM media containing the respective antibody or PBS (input bacteria). Next, these cultures were added over cells monolayers and incubated for 1 h under anaerobic conditions. In this adherence assay, *R. inulinivorans* showed an increased adherence to epithelial cells in the presence of anti-flagellin antibodies compared to non-treated bacteria or treated with the isotype-control antibody (Figure 11A), which is concordant with the results obtained with the non-flagellated strains. These results strongly suggest that the flagellum is not required for adhesion to epithelial cells in our model and, indeed, the flagellum loss in our mutants increases by an unknown manner the adherence to this intestinal component.

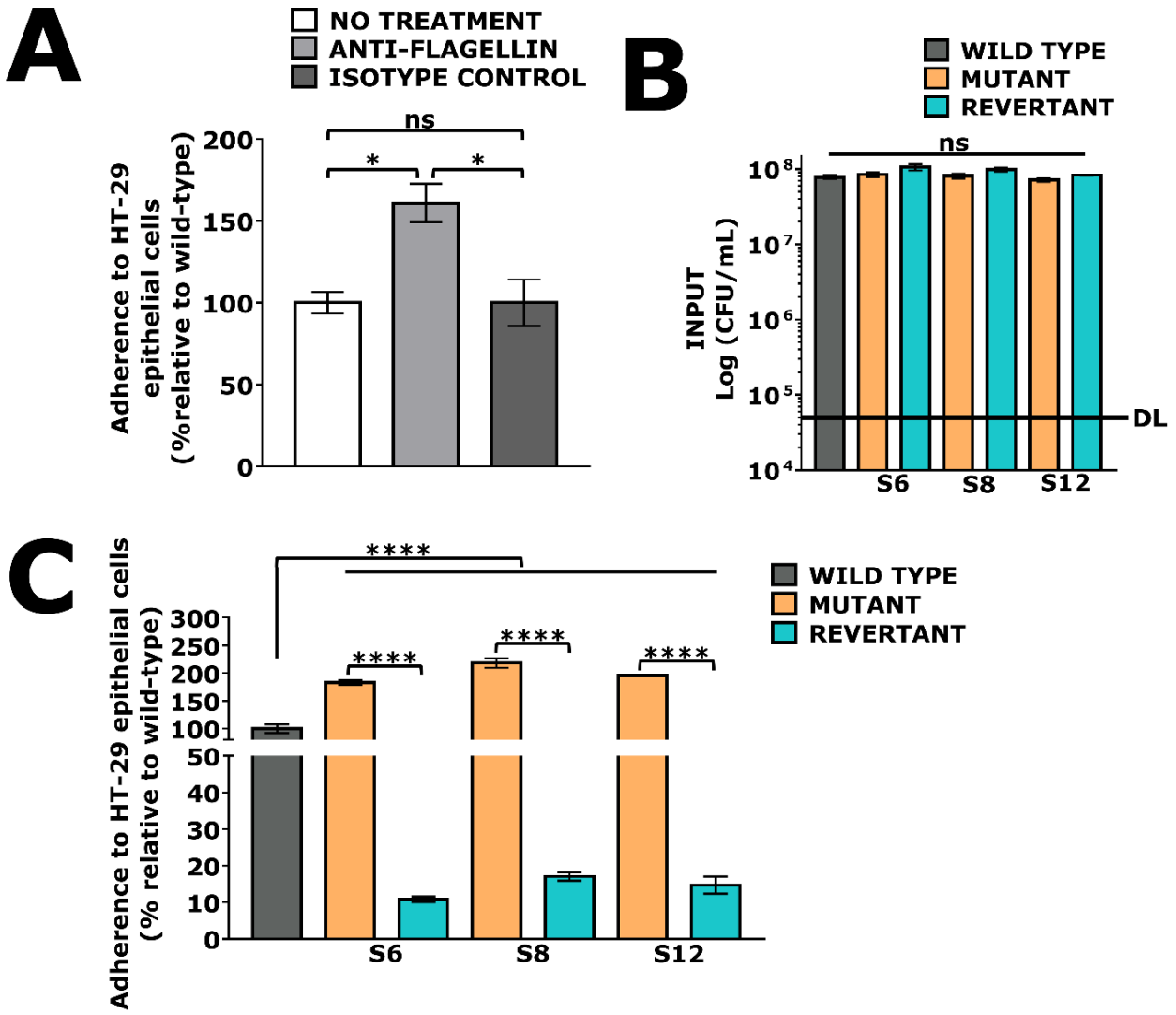


Figure 11. Adherence to epithelial cells *in vitro*. Adherence to epithelial cells was determined *in vitro* in HT-29 cell monolayer; (A) A culture of wild-type strain in YCFA-CGM broth grown 4 h was treated for 2 h more with anti-flagellin antibody or isotype-control antibody in YCFA-CGM broth, then cultures were resuspended in DMEM containing the respective antibody and added to wells of 96-well plates over cell monolayers. Then, after 1 h, wells were washed 3 times with PBS and adhered bacteria were recovered and seeded on YCFA-CGM plates to determine CFU/mL (Representative figure of N=3, differences were determined on adherence percentage relative to the input, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; error: SEM). Log phase cultures (6 h) growing in YCFA-CGM broth were rinsed 3 times in PBS. Cultures were resuspended in DMEM and these suspensions were seeded in YCFA-CGM plates to determine input CFU/mL (B) (Representative figure of N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; error: SEM, DL: Detection limit), then cultures were resuspended in DMEM and added to wells of 96-

well plates over cell monolayers, 1 h after wells were washed 3 times with PBS, and adhered bacteria were recovered and seeded on YCFA-CGM plates to determine CFU/mL (C) (Representative figure of N=3, differences were determined on adherence percentage relative to the input, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; error: SEM).

8.7. Flagellum relevance in adherence to mucin.

Another intestinal component to which bacteria in the gut adheres is the mucus layer, which covers the epithelial barrier in the intestine (M. E. V. Johansson et al., 2011). The mucus layer is a gel-like matrix composed of mucins which are adhesins substrates for many bacteria (Tsilia et al., 2015). In this matrix, the flagellum could not only participate as an adhesin but also as a propeller allowing bacteria to move inside. To evaluate whether flagellum is essential for adherence to the mucus layer in *R. inulinivorans*, an *in vitro* model of this gel-like matrix was used. As in the adherence assay to epithelial cells, bacteria were grown in YCFA-CGM broth for 6 h (input bacteria). At this point, bacteria were directly added to mucin-agar (5% p/v - 0.5% p/v) matrix in 24-well plates and incubated under anaerobic conditions. After 45 min, wells were washed with PBS, and bacteria were recovered in PBS by mechanical disruption of the matrix (output bacteria). CFUs/mL recovered from mucin agar were lower in mutants than the wild-type or revertant strains, while the last two have similar adherence percentages (Figure 12C). When adhesion to the mucin-agar matrix was performed in the presence of an anti-flagellin antibody in the wild-type strain, a slight reduction was observed in adherence compared to the untreated culture or isotype-control antibody-treated culture (Figure 12A). These results indicate that the flagellum is required for adherence to mucins.

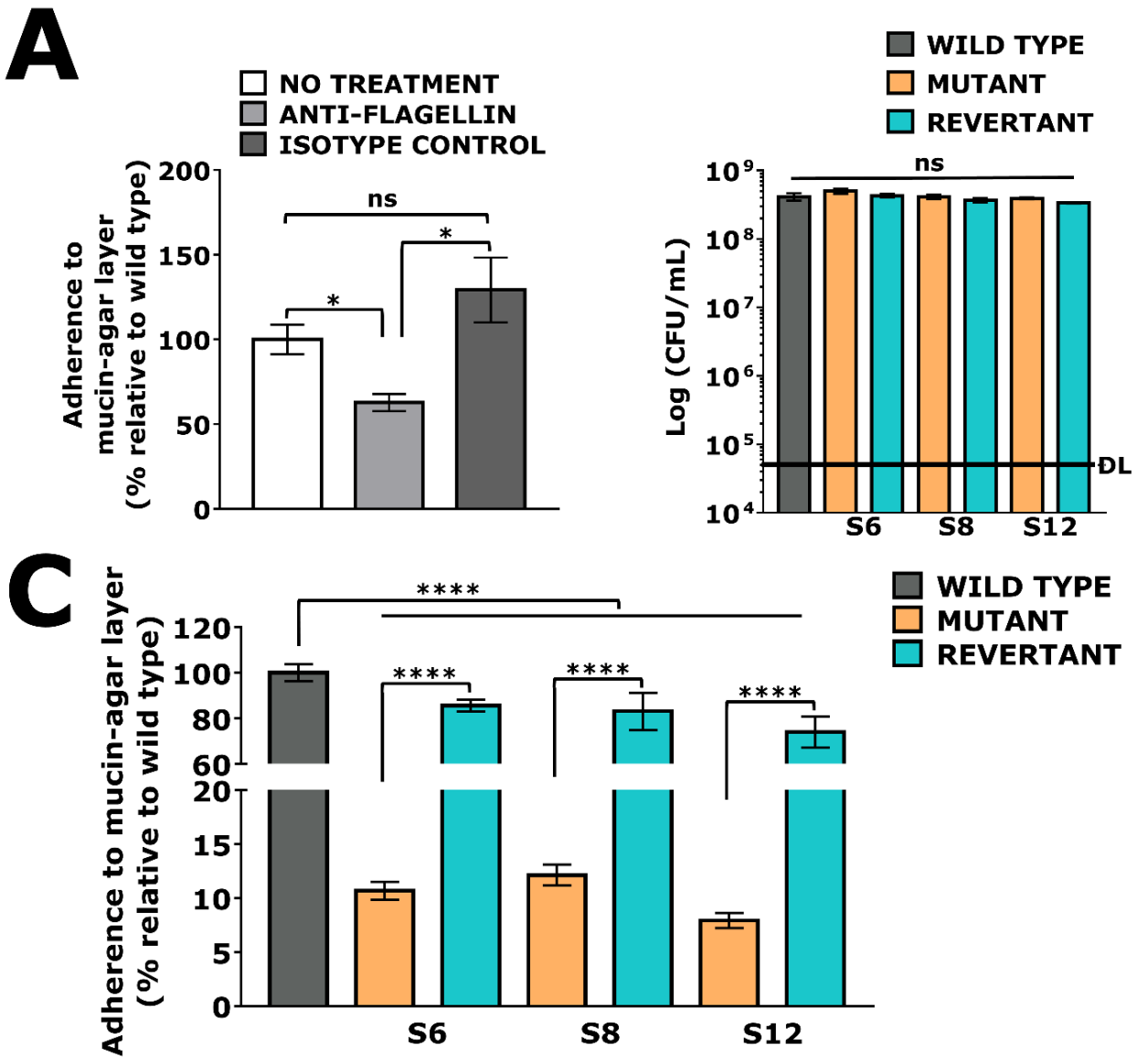


Figure 12. The adherence mucus layer *in vitro*. Adherence was determined *in vitro* in mucin-agar layers. (A) A culture of wild-type strain in YCFA-CGM broth grown 4 h was treated for 2 h more with anti-flagellin antibody or isotype-control antibody in YCFA-CGM broth. Cultures were added to wells of 24-well plates over mucin-agar layer, 45 min after wells were washed 3 times with PBS, and adhered bacteria were recovered and seeded on YCFA-CGM plates to determine CFU/mL (Representative figure of N=3, differences were determined on adherence percentage relative to the input, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; error: SEM). Log phase cultures growing in YCFA-CGM broth were rinsed 3 times in PBS, then were seeded in YCFA-CGM plates to determine input CFU/mL (B) (Representative figure of N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; error: SEM, DL: Detection limit), then cultures were resuspended in DMEM

and added to wells of 24-well plates over mucin-agar monolayers, 1 h after wells were washed 3 times with PBS, and adhered bacteria were recovered and seeded on YCFA-CGM plates to determine CFU/mL (C) (Representative figure of N=3, differences were determined on adherence percentage relative to the input, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; error: SEM).

8.8. Biofilm formation.

Biofilm formation is a related phenotype to motility and adherence (Wu et al., 2020). Biofilms are microbe communities attached to surfaces surrounded by an extracellular matrix of polymeric substances. The biofilm formation can be assessed by crystal violet staining in microtiter plates and determined by optical density at 570 nm (OD_{570}) (O'Toole, 2011). Thus, *R. inulinivorans* strains were grown overnight, and the culture was diluted 1:40 in fresh YCFA-CGM broth, these bacterial suspensions were dispensed in 96-well plates and incubated for 24 h. After crystal violet staining, OD_{570} was higher than the wild-type in Mut6, Mut8, and Mut12, which suggests a higher biofilm formation (Figure 13A), even when Mut6 has a lower number of bacteria in the biofilm (Figure 13A). Conversely, revertant strains Rev6, Rev8, and Rev12 have lower biofilm formation than the wild-type strain (Figure 13B). These results suggest that loss of flagellum in *R. inulinivorans* generates an increase in biofilm formation, which correlates with adherence to epithelial cells.

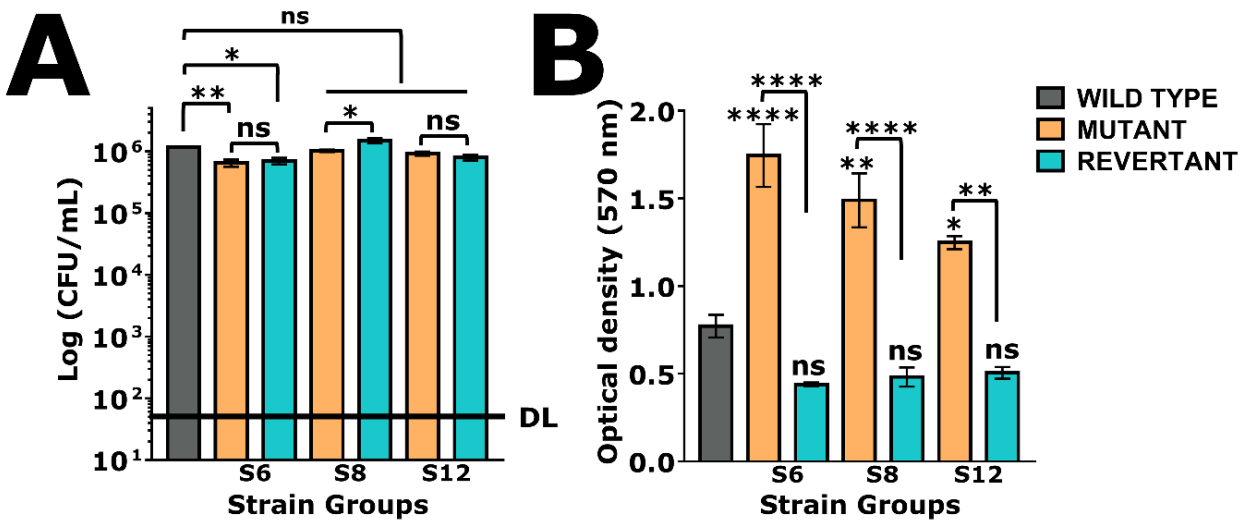


Figure 13. Biofilm formation. Overnight cultures of each strain were diluted 1:40 in fresh YCFA-CGM broth and incubated 24 h in 96-well plates. Then each well was washed twice with PBS to remove pelagic bacteria (*i. e.*, not in the biofilm). After, 3 wells of each strain were resuspended in PBS to determine CFU/mL in the formed biofilm (A) (Representative figure of N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; $p<0.005$, **; error: SEM, DL: Detection limit). Next, biofilm formation was evaluated by the crystal violet staining method (B) (Representative figure of N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.005$, **; $p<0.0001$, ****; error: SEM. Comparison with the wild-type strain is indicated over each column). For both figures, strain groups (S) include the mutant (orange) with its respective revertant (turquoise), wild-type is colored grey.

8.9. Development of the colonization model.

The two colonization models used to study the contribution of defined bacterial species or consortia are germ-free and antibiotic-treated mice (Elizabeth A. Kennedy, 2018). Therefore, both models allow the introduction of new bacterial species and evaluate the colonization process of each bacterial species. While germ-free has no inherent colonization resistance, in antibiotic-treated mice colonization resistance is diminished by a reduction of bacterial abundance in the intestine. To determine whether under our conditions the bacterial load in the intestine of conventionally-raised C57BL/6 mice could be reduced, 3 mice were treated for 14 days with VKAM antibiotic cocktail, and CFUs/g were determined every 7 days in YCFA-CGM plates under aerobic and anaerobic conditions. Figure 14 shows that 7 days is enough to significantly reduce the bacterial load in feces in both anaerobic and aerobic conditions (Figure 14A and 14B, respectively).

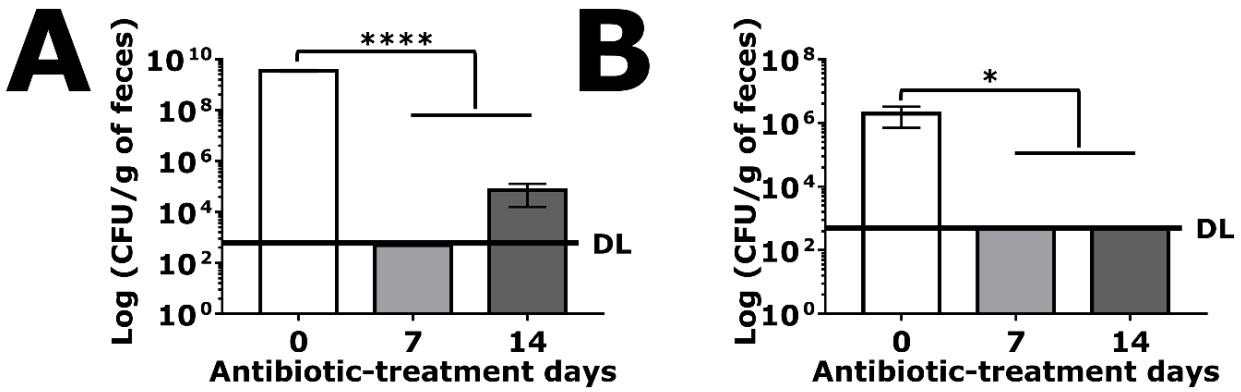


Figure 14. Reduction of bacterial load in feces by VKAM antibiotic cocktail. Conventionally-raised 8 weeks old mice were treated with an antibiotic-cocktail solution in PBS containing vancomycin (50 mg/Kg), kanamycin (100 mg/Kg), ampicillin (100 mg/Kg), and metronidazole (10 mg/Kg) for 14 days via oral gavage. At days 0, 7, and 14 bacterial loads in mice feces were determined by seeding serial dilutions of freshly obtained feces in YCFA-CGM. After 72 h, CFUs were counted and Log(CFU/g of feces) were determined for each day (Mice, N=3, ANOVA with Tukey's multiple comparison test as *post hoc*; ns, no significative; $p<0.05$, *; $p<0.0001$, ****; error: SEM).

One limitation of antibiotic-treated mice is that microbiota depletion is not achieved. Moreover, small communities of bacteria can still be present after antibiotic treatment, which impairs colonization studies of species that generally inhabit the microbiota of mice. It has been observed that in the microbiota of conventionally-raised C57BL/6 mice, *Roseburia* is a core genus and, even more relevant, *R. inulinivorans* closely related operational taxonomic units have been detected in these mice (Septyaningtrias et al., 2020; Wang et al., 2019). Then it was necessary to generate a strategy to evaluate the colonization of the non-flagellated mutant species in our model without interfering with *Roseburia* species in mice microbiota. It has been previously demonstrated that *R. inulinivorans* A2-194 (the same used in this study) can be transconjugated with pMTL83151 plasmid that autonomously replicates inside this bacterium (Sheridan et al., 2019). This plasmid carries the *catP* gene which code for an enzyme that gives chloramphenicol/thiamphenicol resistance to bacteria. Thus, wild-type and non-flagellated mutants Mut8 and Mut12 were transconjugated with pMTL83151 plasmid. Next, it was determined that the solid-medium MICs for thiamphenicol in YCFA-CGM plates for wild-type, Mut6, Mut8, and their respective transconjugants strains, and were included in these MICs freshly obtained feces resuspended in PBS to determine the thiamphenicol concentration that allows the growth of the transconjugant strains and avoid the growth of

bacteria from mice feces. It was observed that non-flagellated mutants did not grow at any concentration evaluated of thiamphenicol (10 µg/mL was the lower concentration), bacteria from feces grew in YCFA-CGM plates until 40 µg/mL, and transconjugant grew until 100 µg/mL (Table 5).

Table 5. Solid-medium growth at increasing thiamphenicol concentrations.

	WT	M6	M8	M12	WT TC	M6 TC	M8 TC	M12 TC	FS
10 µg/mL	-	-	-	-	+	+	+	+	+
20 µg/mL	-	-	-	-	+	+	+	+	+
40 µg/mL	-	-	-	-	+	+	+	+	+
80 µg/mL	-	-	-	-	+	+	+	+	-
100 µg/mL	-	-	-	-	+	+	+	+	-
150 µg/mL	-	-	-	-	-	-	-	-	-

* Letter code: wild-type (WT), Mut6 (M6), Mut8 (M8), Mut12 (M12), wild-type transconjugated (WT-TC), Mut6 transconjugated (M6-TC), Mut8 transconjugated (M8-TC), Mut12 transconjugated (M12-TC), mice fecal suspension (FS). (N=3) (No growth, -; growth, +).

Together these results demonstrate that our antibiotic cocktail administration reduces the bacterial load in feces, which suggests that colonization resistance can be reduced. As well, a concentration of 100 µg/mL of thiamphenicol in YCFA-CGM plates allows the growth of transconjugant strains and avoids the growth of bacteria from mice feces, which suggests that these plates can be used to determine the presence of transconjugant *R. inulinivorans* strains and to eliminate the background species of mice microbiota present in feces. Thus, our model to evaluate colonization in *R. inulinivorans* is shown in figure 15.

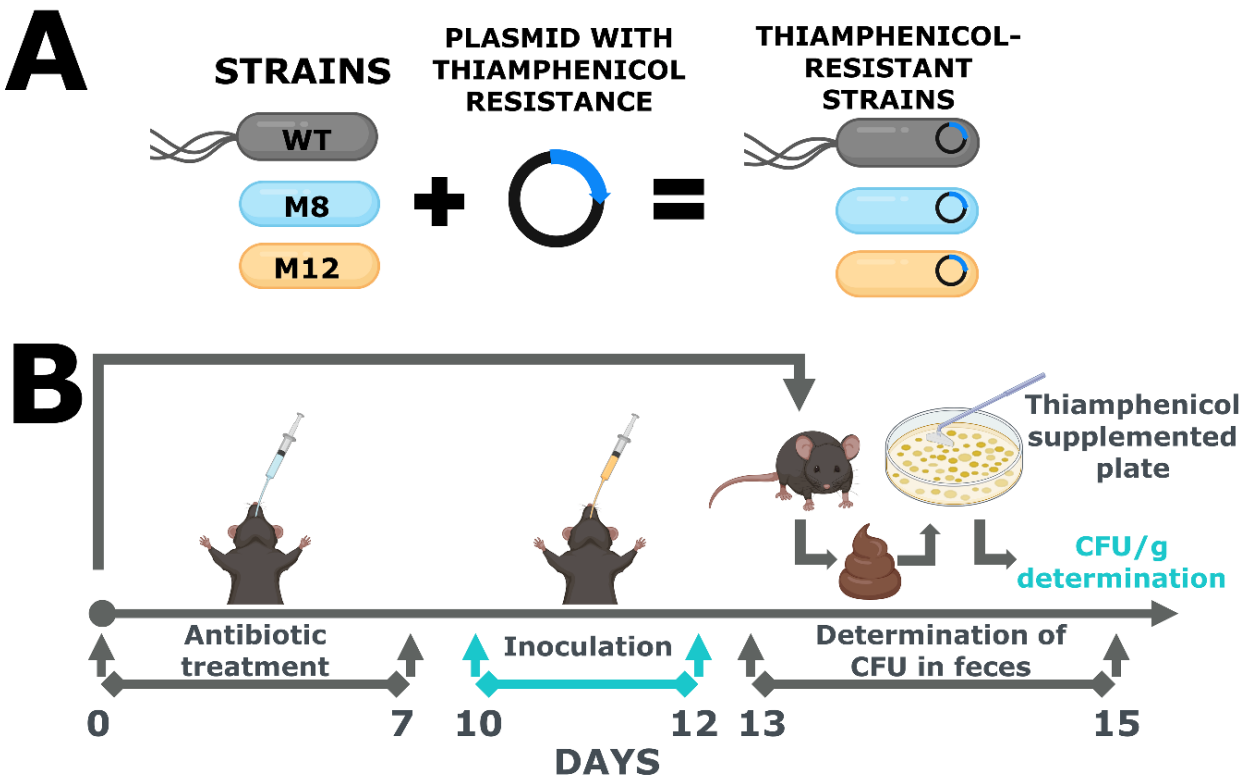


Figure 15. Model for determination of *R. inulinivorans* colonization. (A) Previous generated mutant strains Mut8, Mut12, and the wild-type strains were transconjugated with pMTL83151 plasmid to acquire thiamphenicol resistance. (B) 8 – 12 old weeks were treated for 7 days with VKAM antibiotic cocktail via oral gavage. Then, on the 3rd-day post-antibiotic treatment mice were inoculated with 10^8 CFU for 3 consecutive days and, after the last inoculation, mice feces were collected daily for 3 consecutive days and seeded in YCFA-CGM plates supplemented with 100 µg/mL of thiamphenicol. Finally, CFUs/g of feces were determined in YCFA-CGM plates at 72 h of plate incubation (CFUs/mL were also evaluated at the beginning of the assay, day 0, and the day before inoculations, day 9).

8.10. Relevance of flagellum in the colonization of *R. inulinivorans*.

The flagellum is a complex molecule structure whose principal function is to allow motility in different environments for many bacteria, but it also participates as an adhesin to surfaces. Both functions are relevant for colonizing the gut for many bacteria, mainly pathogenic, while for others, the flagellum is not necessary (Baban et al., 2013; Chaban, Hughes, & Beeby, 2015). To determine whether flagellum is relevant for the colonization o *R. inulinivorans*, an antibiotic-treated mice model was used. Thus, 18 animals (8 – 12 weeks,

male and female) were caged individually and treated for 7 days with VKAM antibiotic cocktail and separated randomly into 4 groups, wild-type inoculated, N=5; Mut8 inoculated, N=5; Mut12 inoculated, N=5; and non-inoculated mice, N=3. Two days after the end of antibiotic treatment, mice feces were seeded in YCFA-CGM plates supplemented with thiamphenicol (100 µg/mL), and no CFUs were observed in any mice feces after 72 h of incubation under anaerobic conditions (Data not shown). On the third day after the end of antibiotic treatment, mice were inoculated with 10^8 CFUs of the respective strain or YCFA-CGM broth (no inoculated mice) for 3 consecutive days; then, 12, 36, and 60 h post inoculations (PIs) mice feces were collected and seeded in YCFA-CGM thiamphenicol-supplemented (100 µg/mL) plates. After 72 h of incubation, thiamphenicol-resistant CFUs were counted and Log(CFUs/g) were determined. On day 1 and 2 PIs, 4 mice presented *R. inulinivorans* colonies, 3 from Mut8 inoculated group and 1 from Mut12 inoculated group (Figures 16A and 16B). At day 3 PIs (Figure 16C), only 2 mice from Mut8 inoculated group presented *R. inulinivorans* colonies. In summary, no *R. inulinivorans* colonies were detected on the 3 days PIs evaluated when mice were inoculated with the flagellated wild-type strain. By contrast, when mice were inoculated with non-flagellated mutants, 3 of 5 mice inoculated with Mut8 and 1 of 5 mice inoculated with Mut12, *R. inulinivorans* was detected in feces (Figure 16D). These results suggest that non-flagellated strains colonize better than the wild-type strain in our colonization model.

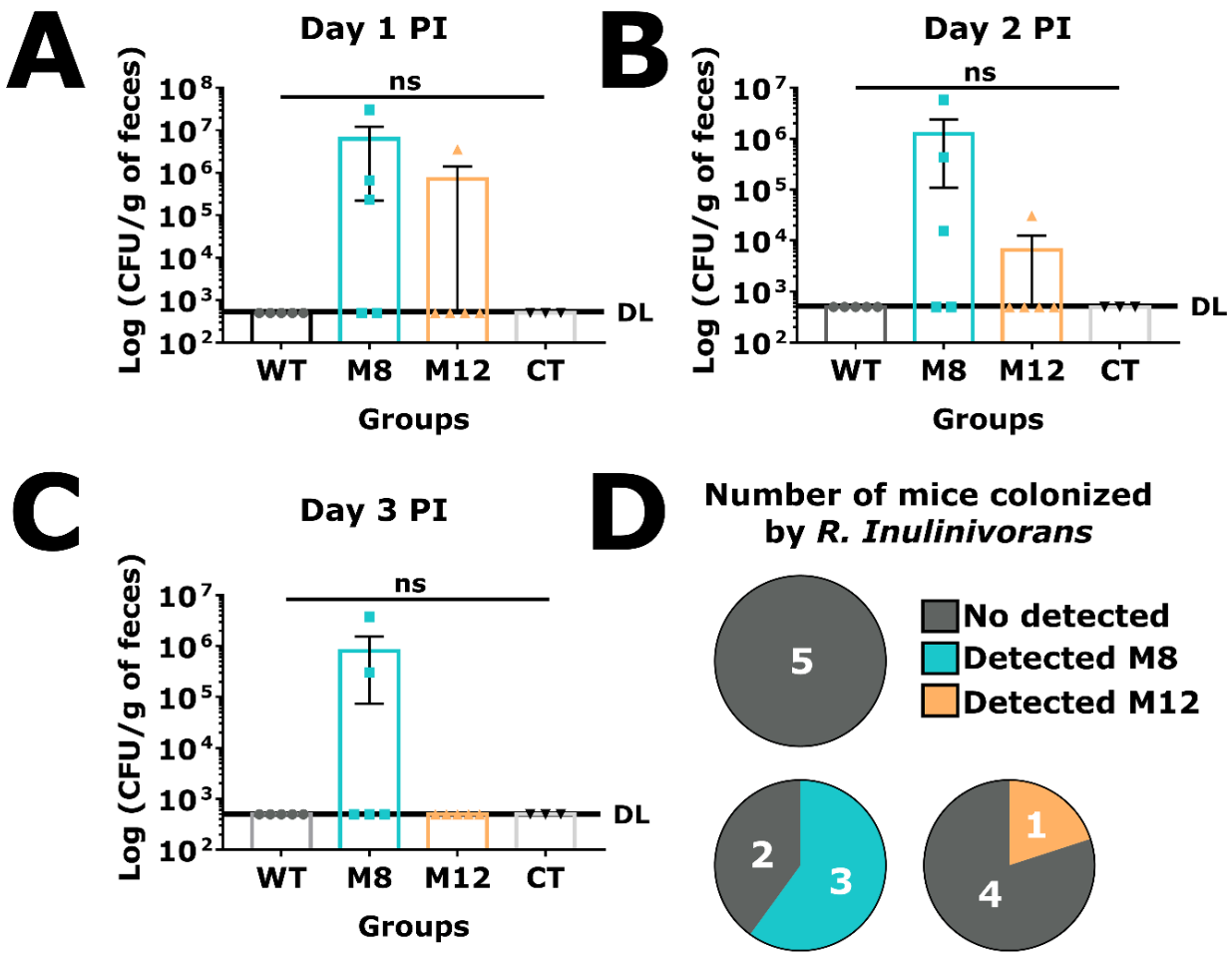


Figure 16. Colonization of *R. inulinivorans* strains in mice. Colonization assays were performed in conventionally-raised mice treated with VNAM antibiotic cocktail for 7 days. Mice were inoculated with 10^8 CFU of thiamphenicol-resistant strains (WT-TC, Mut8-TC, and Mut12-TC) for three consecutive days. Then, feces were collected from 1 to 3 days post-inoculation and seeded in YCFA-CGM plates supplemented with a thiamphenicol concentration that avoids the growth of normal microbiota of mice. After 72 h incubation CFUs in plates were determined at day 1, 2, and 3 post-inoculation, figures A, B, and C, respectively (Mice, N=5 per group for WT, M8, and M12; N=3 for CT group; Kruskal-Wallis with Dunn's multiple comparison test as *post hoc*; ns, no significative; error: SEM, DL: Detection limit). D, the number of mice colonized by *R. inulinivorans* strains on each group; wild type, upper; Mut8, bottom left (number of colonized in turquoise); Mut12, bottom right (number of colonized in orange).

9. DISCUSSION

R. inulinivorans is one of the five species of an important genus of the human gut microbiota, comprising from 3 to 15% of the total bacteria detected in feces. Therefore, studying its colonization mechanisms is relevant, but no genetic tools for site-directed mutagenesis have been developed yet for this genus which generates an impairment in the search for genetic determinants of the colonization process.

9.1. Chemical mutagenesis and spontaneous reversions of *R. inulinivorans*.

Mutagenesis has been a valuable tool for understanding genetic determinants or proteins implicated in diverse processes. There are many ways to generate mutagenesis in organisms, from sophisticated site-directed mutagenesis strategies to random mutagenesis. The last has been used for decades when site-directed mutagenesis was unavailable for a specific organism (Liwerant, 1975; Shelake, Pramanik, & Kim, 2019). Chemical mutagenesis has been widely used and many chemical mutagens have been described. One of them is EMS, an inorganic solvent that produces random mutations in DNA principally by guanine alkylation and produces a nucleotide substitution that may generate transition mutations (GC to AT and AT to GC) (Sega, 1984), which were found in our study in many genes including flagellar machinery genes (Table 4, Table S1, Figures 9, and Figure 10). EMS treatment also causes base-pair insertion or deletions and large intragenic deletions (Sega, 1984), also observed in *R. inulinivorans* after the treatment with EMS (Table S1). As a mutagen, this compound is also toxic, therefore increases in concentration generate a concomitant decrease in the survival of bacteria (Figure 2A). The effect of EMS concentration on the mutational rates can be estimated by the appearance of rifampicin-resistant mutants since single base mutations on the *rpoB* gene generate this resistance (Jin & Gross, 1988). It was observed that rifampicin-resistant mutants increased directly with the EMS concentration from 2.5 to 20 m/mL in *R. inulinivorans* (Figure 2B), and the lowest was selected to induce non-flagellated mutants, which generated 19 no-motile strains from which 7 were confirmed for flagellum loss. Genome analysis of the 7 non-flagellated strains showed that 15 mutations per genome were generated on average (12 to 19 mutations per genome) after EMS treatment at 2.5 mg/mL, which includes single-base substitutions, single-base deletions, and deletions (Table S1). It was observed that some mutant strains (Mut4, Mut9, and Mut13) present higher t_d at 6, 9, and 12 h (Table S2) which suggests that mutations in these strains were generated in relevant genes for the survival or fitness of *R. inulinivorans* but were not determined because these strains were not sequenced.

When a gene has been mutated, the phenotype can be restored by true or pseudo reversions through multiple generations. A true reversion is a restoration of the genome sequence, while pseudo reversions are all the new mutations in the genome that re-establish the phenotype (Rosenberg, 2013). After multiple passages in semi-solid YCFA-CGM plates, only 7 of 19 non-motile mutants have reverted to the motile phenotype after 60 generations. This suggests that reversion in the other 12 strains is more complicated possibly for mutations as deletions affecting more than 2 bp in flagellar-machinery genes, that have lower reversion rate when compared to single-base substitutions or single-base insertions/deletions. This is plausible since mutations larger than 10 bp were detected in the genome of some mutants (Table S1).

9.2. Motility differences are generated for the flagellum loss.

Motility is the concerted movement of cells by self-propulsion and is widespread among bacterial species. Different types of motilities include twitching, gliding, sliding, swarming, and swimming, where different molecular structures participate (Wadhwa & Berg, 2022). One structure that participates in motility is the flagellum, specifically in swarming and swimming motility. The flagellum is a complex structure composed of approximately 25 proteins divided into three parts, the FBB, the hook, and the filament (H. Hu et al., 2022). Flagellar motility can be modified by mutations in structural or accessory genes, which can produce both an impairment or an increase in this phenotype, as observed for mutants and revertants obtained for *R. inulinivorans*, respectively (Figure 7). As mentioned before, all non-motile strains evaluated by TEM were non-flagellated, even when the non-motile phenotype is generated by two phenomena, flagellum loss, and paralyzed-flagellum, and it was expected to observe both types of phenotypes (Smith & Lefebvre, 1997).

9.3. Mutations in the basal body generate flagellum loss.

Three genes were mutated in *R. inulinivorans* that generate non-flagellated phenotype, *flhB*, *fliM*, and *fliP* (Table 4). All these genes code for proteins that are constituents of the FBB, specifically FlhB and FliP are part of the export apparatus, while FliM is a constituent of the C-ring (Johnson et al., 2021; Minamino, Kinoshita, & Namba, 2019). Since nonsense mutations in our mutants generate in most cases, except in Mut19, the loss of more than half of the protein (Table 4) the loss of function is reasonable. However, the mutation sites were analyzed.

Mutation on FlhB generates a stop codon at residue 17 (Mut15 and Mut17). Therefore it generated a short-truncated protein that explains the observed phenotype because it has been observed that deletion of this gene in many bacteria generates the flagellum loss (Cheng et al., 2018; Hendrixson & DiRita, 2003; Meshcheryakov, Barker, Kostyukova, & Samatey, 2013). Surprisingly, the reversion of this mutation was not in this gene because revertants strains maintain this mutation, then pseudo-reversions in a different gene could be implicated in this phenomenon in our strains. Only one mutant strain has shown mutations in the FliM protein, Mut12 has a mutation in the aminoacidic residue 167 of 326, which generates a stop codon that truncates this protein in the middle, FliM is part of the switch complex involved in the control of motor switching between clockwise and counter clockwise rotation, this complex includes proteins FliG and FliN, and all three proteins of the complex are required for flagellum assembly (Mathews, Tang, & Blair, 1998). In *E. coli* has been observed that FliM links to itself and the other two proteins of the complex, and FliG can interact with multiple residues of FliM, while for FliM-FliN interaction is necessary a C-terminal region of 90 residues. It has been demonstrated that when the interaction of FliM-FliN is abolished flagellum is not assembled and a non-flagellated phenotype is observed, which explains the loss of flagellum by Mut12 which has lost the last 159 C-terminal residues of FliM, that even if is stable and not degraded cannot interact with FliN (Mathews et al., 1998). Since Rev12 has generated a true reversion and flagellated phenotype was restored, the functionality and relevance of FliM in the assembly of the flagellum in *R. inulinivorans* are strongly suggested. Finally, mutations in FliP were observed in four strains (Mut6, Mut8, Mut14, and Mut19). Three present the same nonsense mutation in residue 126 and the other has a nonsense mutation in residue 270 of 282 amino acids length. The first mutation generates the loss of most than half of the protein which explains the observed phenotype. FliP forms a complex with FliQ and FliR, where multiple copies of each protein are together and generate a channel in the export apparatus. As observed in other species, FliP forms a homohexamer ($FliP_6$) that initiates the complex FliPQR formation and is necessary for protein export and flagellum assembly (Fukumura et al., 2017). For the formation of the $FliP_6$ ring, a region of 6 residues in the middle of the protein (from amino acid 150 to 160) are necessary because it allows the interaction of FliP-FliP and the interaction of FliP with FliO, an accessory protein that participates in the assembly of $FliP_6$ (Barker, Meshcheryakova, Inoue, & Samatey, 2014; Fabiani et al., 2017; Fukumura et al., 2017). Thus, even if truncated FliP is stable cannot self-interact forming the $FliP_6$ homohexamer or FliPQR complex, which explains the flagellum loss of the first mutation. As the second mutation is closer to the C-terminal region of FliP and no evidence exists of the

functionality of this region, the mutation could generate an unstable and degraded protein. Reversion of the four FliP mutant strains occurs in different ways, but only reversions in Rev6, Rev8, and Rev19 can be explained by mutations observed in the flagellar machinery because Rev 14 maintains the mutation of Mut14. Rev19 reversion eliminates the stop codon but generates a nonsynonymous mutation compared to the wild type. This confirms that the mutation in Mut19 generates the flagellum loss in *R. inulinivorans*. Interestingly Rev6 and Rev8 strains revert the same mutation observed in Mut6 and Mut8 in different ways. Rev8 has replaced the nonsense mutation for a missense mutation, while Rev6 has reverted the phenotype through a single-nucleotide insertion that generates a frameshift and a truncated protein of 155 amino acids but 29 amino acids larger than FliP of Mut6 (Figure 17). The reversions generated in Rev6, Rev8, and Rev19 demonstrate that FliP is functional and necessary for the flagellated phenotype in *R. inulinivorans*.

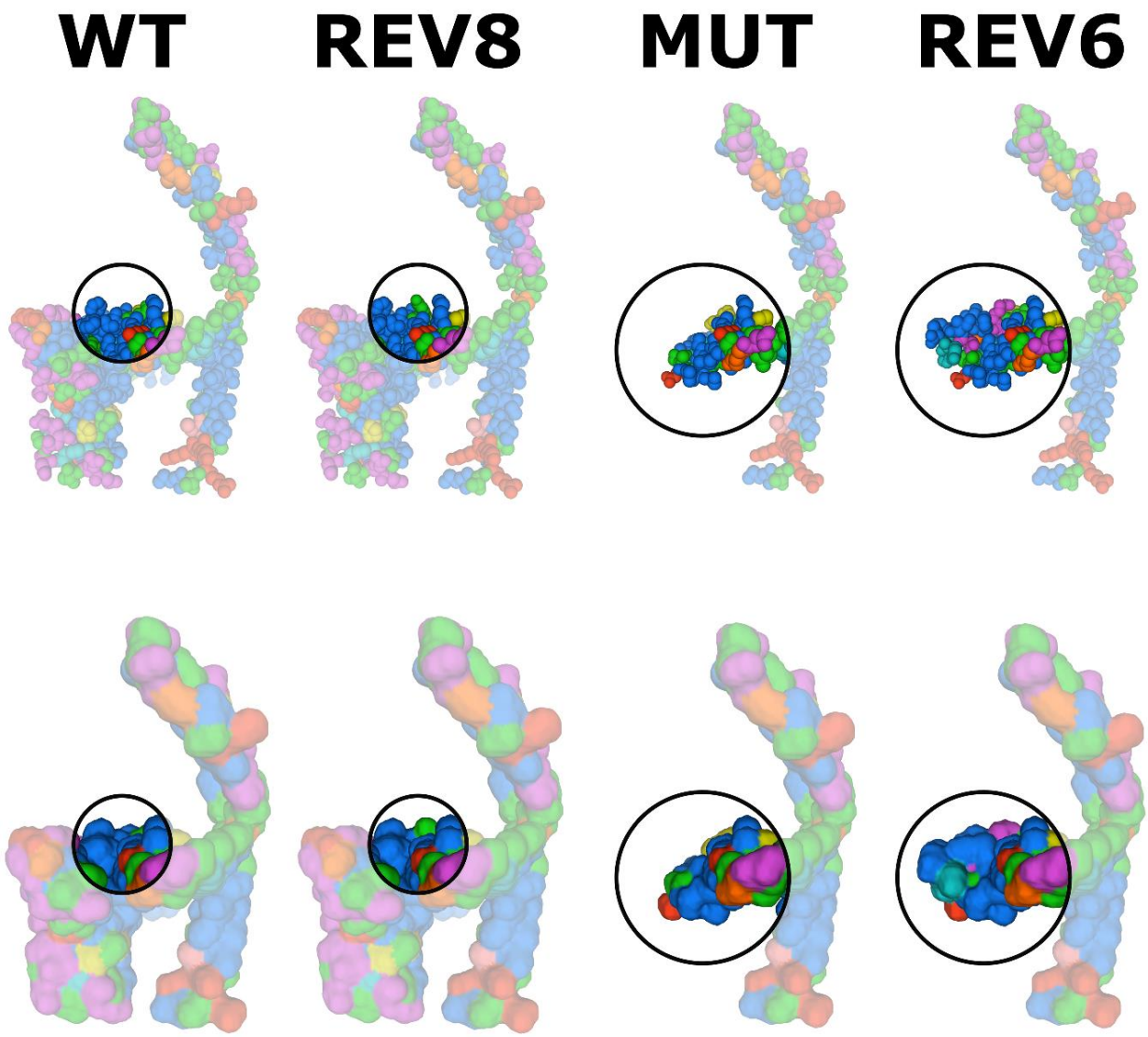


Figure 17. Protein modeling of FliP by homology in *R. inulinivorans* strains. Aminoacidic sequences of FliP wild type, Rev6, and Rev8 were used to model protein structure on Swiss-Model online software. The predicted changes in protein structure are in circles in both Spacefill (upper figures) and Surface (bottom figures). The template used for modeling was the Flagellar biosynthetic protein FliP of R. inulinivorans (UniProtKB ID A0A173RK80).

9.4. The higher motility in revertant strains

Enhancement of flagellar motility could be the product of mutations in diverse genes that do not necessarily have to be directly related to flagellar machinery structure or regulation (Ni et al., 2017).

After multiple generations bacteria could accumulate multiple mutations in their genome, generating changes in one or more phenotypes. As previously mentioned only mutants Mut6, Mut8, Mut12, and Mut19 have reversions that could explain the reversion to flagellated phenotype. These strains have 5, 4, 6, and 11 specific mutations, respectively (Table S3). From these mutations, only Rev6, Rev8, and Rev12 have mutations related to flagellar motility. Rev12 has a nonsense mutation in the C-terminal of FliF, the protein that forms the MS-ring and interacts with FliG (Figure 1) in the rotor of the FBB. The interaction FliF-FliG occurs in the C-terminal region of FliF (Levenson, Zhou, & Dahlquist, 2012), therefore in these revertant strains the interaction between these two proteins could be altered in some way, but more studies have to be done to confirm this hypothesis. Interestingly, as mentioned before, Rev6 and Rev8 have a mutation in CheY. This chemotaxis regulatory protein determines the rotation direction of the flagellum when it is bound to FliM in either phosphorylated or unphosphorylated forms, which generates a clockwise (CW) or counterclockwise (CCW) rotation, depending on the specie (Nakamura & Minamino, 2019). Exist an equilibrium between CheY-CheY~P concentration determined for the autophosphorylation, or phosphorylation mediated for CheA, and for dephosphorylation that occurs dependent or independent of CheZ. Thus, CheY interacts with these 3 proteins, FliM, CheA, and CheZ to determine the flagellum rotation and generate a smooth (CCW rotation) or tumbled motility (CW rotation). Rev6 and Rev8 have the nonsynonymous mutations D99E and A113E (Figure 9). At these positions both CheA and CheZ can bind in another bacteria, then mutations in this portion of the protein and its phosphorylated state change the equilibrium CheY-CheY~P to an equilibrium that generates a CCW rotation with the concomitant smooth motility (Zhu, Volz, & Matsumura, 1997). As shown in figure 18, when modeled, these mutations change the same zone of the 3D protein structure. Thus, it is probable that both Rev6 and Rev8 mutations on CheY protein increase the unphosphorylated form generating continuous or mostly smooth motility observed as higher motility in these strains. Further studies need to be made to confirm that these mutations produce the observed phenotypes.

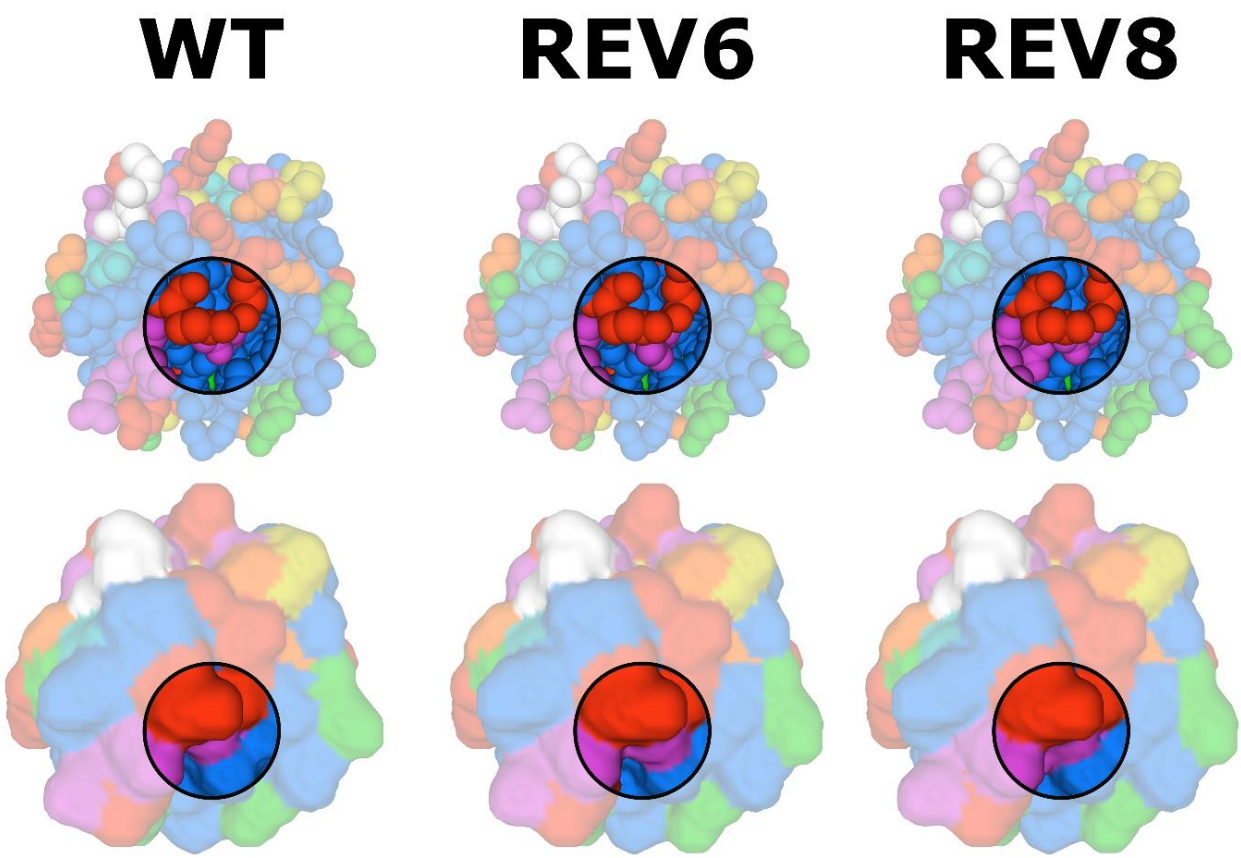


Figure 18. Protein modeling of CheY by homology. Aminoacidic sequences of CheY wild type, Rev6, and Rev8 were used to model protein structure on Swiss-Model online software. The predicted changes in protein structure are in circles in both Spacefill (upper figures) and Surface (bottom figures) models. The template used for modeling was Structure of phosphono-CheY from *Thermotoga maritima* (ID 4qyw.1) which presents the higher Global Model Quality Estimate (GMQE) score.

9.5. The flagellum of *R. inulinivorans* participates in adherence to the mucus layer but impairs the adherence to epithelial cells.

The adherence as a colonization factor has been studied principally on intestinal pathogenic bacteria, where bacteria can interact and adhere with constituents of the intestine as the mucus layer and the epithelial cells by different mechanisms and structures (Juge, 2012; Terashima et al., 2008). Adherence to the mucus layer and epithelial barrier can be simulated and studied *in vivo* (Figure 11 and Figure 12) (McKee et al., 2018; Van den Abbeele et al., 2009). Even when most studies have been done on pathogenic bacteria due to the relevance of adhesion in the infectious process, some studies have reported the adhesion in

gut microbiota bacteria. In the *Bifidobacterium* genus, pili have been described as an adhesion factor to cell line cultures and contribute to bacterial colonization and persistence *in vivo*, *Bifidobacterium* can also use adhesin-like factors to adhere to intestinal components, as surface-exposed proteins (Ruiz, Delgado, Ruas-Madiedo, Margolles, & Sánchez, 2016). Other *in vitro* studies have shown that *Bacteroides fragilis*, a member of the Bacteroidetes phylum, can adhere to epithelial cells and mucins (Huang et al., 2011). In members of the Firmicutes phylum; such as *F. prausnitzii*, *R. intestinalis*, and *Eubacterium rectale*, among others; adherence to the mucin layer was also demonstrated *in vitro* (Van den Abbeele et al., 2013).

The flagellum acts as an adhesin and propeller that allows the movement from harmful or nutrient-limited zones to safer or nutrient-rich zones. In the adherence, the flagellum mainly participates principally by the filament, where the flagellin protein FliC and the cap protein FliD have been recognized as adhesin proteins that adhere to epithelial cells and mucins (Baban et al., 2013; Girón et al., 2002; Haiko & Westerlund-Wikström, 2013; Rabaan et al., 2001) The relevance of the flagellum in adherence has been poorly studied in members of the human gut microbiota, probably because most of the relevant members of the more abundant phyla are non-flagellated bacteria. One study showed that bacteria from *Clostridium* cluster XIVa, which includes the *Roseburia genus*, can adhere to mucin *in* vitro (Van den Abbeele et al., 2013). Here we studied the role of *R. inulinivorans* flagella in this process *in vitro*; we found that flagellum is relevant for adherence to mucin (Figure 12), an important process since these bacteria belong to a bacterial family that colonizes the mucus layer principally (Donaldson et al., 2016). Conversely, in epithelial cells *R. inulinivorans* increase the adherence when the flagellum is not assembled (Figure 11), which could be explained by the increase of cyclic diguanylate (c-di-GMP), a typical second messenger in bacteria that can accumulate in the bacterium when flagellum assembly is abolished (Wu et al., 2020), that has been observed regulates the epithelial cell adherence in bacteria positively (J. Hu et al., 2013; McKee et al., 2018). Even when we did not evaluate c-di-GMP concentrations we determine the biofilm formation, another factor that increases when c-di-GMP accumulates in the bacterium. As observed in figure 13, non-flagellated *R. inulinivorans* showed a higher biofilm formation than wild-type or revertant strains, which suggests c-diGMP is accumulated in our non-flagellated strains explaining the higher epithelial-adherence phenotype.

9.6. Is the flagellum a colonization factor for *R. inulinivorans*?

The flagellum is a colonization factor for pathogenic bacteria participating actively in virulence (Haiko & Westerlund-Wikström, 2013), conversely, the study of colonization by flagellated members of the human gut microbiota has not been extensively studied. In normal individuals, the flagellin is recognized by Tol-like receptors 5 (TLR5) in the intestine, which induces the production of IgA antibodies by B cells (Sharma, Hecker, Walther, Stuckas, & Hiller, 2020). These antibodies can control the growth of flagellated microorganisms, which determines the microbiota composition (Fulde et al., 2018; Leifer et al., 2014). In the same way, it has also been determined that anti-flagellin antibodies can interact in a non-specific species way to control the motility and flagellar expression in *R. intestinalis* (Cullender et al., 2013). Here we have shown that anti-flagellin antibodies from a phylogenetically distant species (*E.* coli) modify both epithelial-cells and mucin adherence in *R. inulinivorans* (Figure 11A and Figure 12A, respectively). Koch & Barton (2013) postulate that if IgA can bind to flagellin-expressing bacteria, this could reduce the fitness of these microorganisms, favoring the outgrowth of microorganisms that do not express flagella. They also postulate that flagellum could be necessary for bacteria colonization during the early stages of human development, particularly at birth when these B cells that produce these specific IgA antibodies are not present, or during other periods that generate disturbances in the gut environment. We have not found significant differences in colonization between the flagellated wild-type strain and our non-flagellated mutants Mut8 and Mut12 (Figure 16A, 16B, and 16C). However, in the model of colonization used (Figure 15), we observed that only in mice inoculated with non-flagellated strains (Mut8-TC and Mut12-TC), *R. inulinivorans* was detected (Figure 16D). This contradicts the previous finding in the *Roseburia* genus, where *R. cecicola* use the flagellum to compete with other bacteria for cecum colonization. However, in that study, the model is a Penta-colonized mouse derived from germ-free mice with no *Roseburia* genus members before *R. cecicola* colonization (Stanton & Savage, 1983a), which is different from our model, which usually has *R. inulinivorans* or related species in the intestine. These findings could also be explained by differences in flagellum role for different species because even between strains, flagellum could participate in different ways, such as observed in *C. difficile* non-epidemic and epidemic strains where flagellum loss affects in different ways the adherence to epithelial cells (Baban et al., 2013). If flagellum is relevant for *R. inulinivorans* colonization is yet to be determined, since our model, which simulates better a normal organism than germ-free mice, shows a tendency but not an answer to this question.

10. BIOTECHNOLOGICAL PROBLEM

10.1. Alternative biotechnological projection.

The *Roseburia* genus includes strictly-anaerobic, Gram-variable bacteria of the gut microbiota related to human health. In healthy adults, the abundance of this group of bacteria is from 3 to 15% of the total bacteria present in feces, and a decrease in its abundance has been related to diverse diseases. *Roseburia* has been associated with intestinal health through short-chain fatty acids (SCFAs) production, which is involved in intestinal physiology and immune homeostasis. Using carbohydrates as fermentative substrates this genus shows high production of butyrate, a kind of SCFA that is the primary source of energy for epithelial cells in the colon and that has been demonstrated to inhibit the mRNA expression of proinflammatory cytokines in the mucosa. Furthermore, it has been observed that higher butyrate concentrations in the gut are linked to microbiota composition, wherein *Roseburia*/*A. rectalis* group represents 20% of the total bacteria, this could explain the correlation between inflammatory bowel disease (IBD) and the decrease of *Roseburia* abundance in the intestine (Tamanai-Shacoori et al., 2017).

Based on this information, members of the *Roseburia* genus could be used as a probiotic for treating of inflammatory bowel disease if intestinal colonization is achieved.

10.2. Biotechnological problem.

IBD includes two diseases with common characteristics, ulcerative colitis, which affects the larger intestine and rectum with the presence of ulcers, and Chron's disease commonly affects the small intestine to the colon. Both are characterized by intestinal lining inflammation and produce diarrhea, rectal bleeding, abdominal pain, fatigue, and weight loss. Complications may include colon cancer, a higher risk of blood clot formation, bowel obstruction, and toxic megacolon, among others. The prevalence rate for this disease has increased worldwide from 79.5, in 1990, to 84.3, in 2017, per 100,000 population (Safiri et al., 2019). The development of new treatments for this disease is needed since current treatments and new treatments have lower remission rates (Lower than 50%) and many present side effects. Based on these findings, combining treatments for this disease has also gained relevance, also because IBD is a multifactorial disease. Most of the treatments focus on the immune system (~90%). In contrast, microbial-based treatments that include prebiotics, probiotics, and fecal transplantation, represent less than 10% (Figure 19), probably

because even when studies are promising they are limited and have not demonstrated significant benefit yet (Falloon & Fiocchi, 2022). Thus, the biotechnological problem to solve is the lack of an effective treatment for IBD.

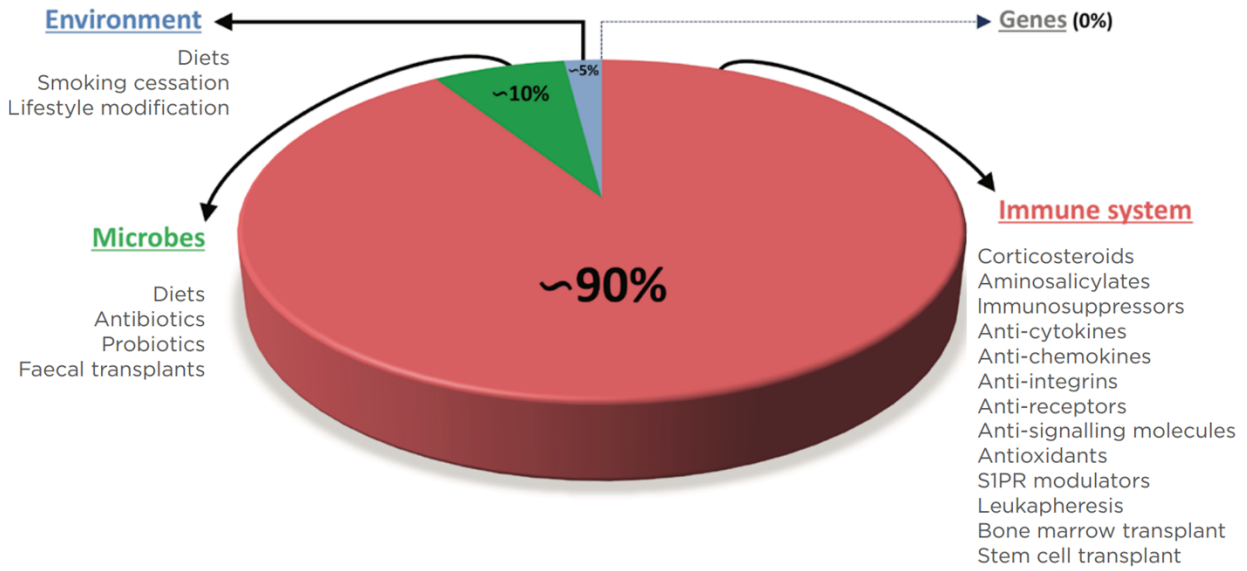


Figure 19. The relative proportion of the different therapeutic interventions available to treat inflammatory bowel disease by targeting the four major pathogenic factors (Falloon & Fiocchi, 2022).

10.3. The solution to the biotechnological problem today.

As mentioned, and shown above (Figure 19), many treatments have been developed and used for IBD. Because our work involved the bacteria *R. inulinivorans*, only patents that include bacteria were considered for the analysis on patent databases, 10 relevant patents are listed below (filled from October 31st, 2002 [20 years from filling]).

- Compositions and Methods for Treating Inflammatory Bowel Diseases (IBDs) and Other Disorders (Finch Therapeutics Holdings Llc): Product and methods.
  - What is claimed in the patent:
  - A Pharmaceutical composition comprising a fecal microbiota including the butyrate-producer genus *Faecalibacterium*, with or without the use of antagonizing species for *Fusobacterium* and/or *Mycobacterium* species. Also, this pharmaceutical composition could include prebiotics and antibiotics. It

is also considered the administration method for treating IBD and other diseases.

- *Roseburia* genus is included in a claim derived from a principal claim.

- Comprise probiotic bacteria, prebiotics component, mineral salt, and lactoferrin compositions (Pfizer Consumer Healthcare Italy SRL): Product and methods.
  - What is claimed in the patent:
    - A probiotic composition that includes *Bifidobacterium longum* and *Lactobacillus rhamnosus*, with lactoferrin addition. These components are in different proportions and include yeast in some claims. It is also considered the administration method for the treatment of intestinal disorders.

- Rapid recolonization deployment agent (Glen Cunningham): Product and methods.
  - What is claimed in the patent:
    - Pharmaceutical compositions which include microorganism mixtures for treating of different gastrointestinal diseases, and the administration of these compositions. Also include a method for microbiota restoration and a kit for insulin resistance syndrome, both with these microorganism combinations.
    - *Roseburia intestinalis* is included in a claim derived from a principal claim.

- Applications of *Bacteroides fragilis* in the prevention and/or treatment of inflammatory bowel diseases (智发朝白杨王晔刘洋洋王从峰): Use and product.
  - What is claimed in the patent:
    - Use of *Bacteroides fragilis* as a probiotic is limited to a specific strain and associated products with this bacterium for treatment or prevention of IBD.

- Use of *Collinsella* for treatment of inflammatory bowel disease (Swecure AB): Methods.
  - What is claimed in the patent:
    - Methods include administering *Collinsella* alone or in combination with other bacteria for treating IBD in mammals (not only for humans).

- Compositions and method for treatment and prophylaxis of inflammatory bowel disease (Ira Milton Trachtman): Methods.
  - What is claimed in the patent:
    - Methods that include antibiotic usage when IBD is generated by pathogenic bacteria, combined with different bacteria combinations treating IBD.

- Method for treatment of disorders of the gastrointestinal system (University of Guelph Queens University at Kingston, Kingston Health Sciences Centre): Product and method.
  - What is claimed in the patent:
    - Synthetic stool preparation that includes a diverse combination of bacteria. It is also considered the administration method for the treatment of gastrointestinal disorders.
    - *Roseburia* genus is included in a claim derived from a principal claim.
    - *Roseburia inulinivorans* is included in a claim derived from a principal claim.

- Compositions comprising co-selected microbiota and methods for use thereof (Nubiyota LLC): Product and methods
  - What is claimed in the patent:
    - An anhydrous composition that includes multiple bacteria combinations and the method for treating mammals with intestinal dysbiosis, including disease as *C. difficile* infection, IBD, etc.
    - *Roseburia* genus is included in a claim derived from a principal claim.
    - *Roseburia inulinivorans* is included in a claim derived from a principal claim.

- Compositions comprising bacterial strains (4D Pharma Research Ltd): Product and method.
  - What is claimed in the patent:
    - Composition that includes a bacterium from the *Roseburia* genus and the administration method for treating or preventing a disease mediated by IL-17 or Th17 pathway.

- Use of microbial communities for human and animal health (Universiteit Gent, Microbial Resource Management Health NV MRM Health): Product and methods.
  - What is claimed in the patent:
    - A composition that includes multiple bacteria combinations and the administration methods to treat different gastrointestinal disorders.
    - *Roseburia* genus is included in a claim derived from a principal claim.
    - *Roseburia inulinivorans* is included in a claim derived from a principal claim.

In conclusion, bacteria used for treating gastrointestinal disorders, including IBD, are based on single bacteria or more commonly the combinations of bacterial species from the normal microbiota as probiotics. In many patents, some bacterial species, genera, or families are shared; the differences are in the methods of administration or composition used to administer these bacteria. The *Roseburia* genus is used in many patents, and some include *R. inulinivorans*. A patent not listed above that exclusively is based on the *Roseburia* genus, which includes *R. inulinivorans*, for human health is:

"Application of *Roseburia* in treating and preventing obesity-related diseases" (BGI Shenzhen Co Ltd).

- This patent is not associated with IBD, but obesity and diabetes, and claim the use of *Roseburia* members in products, such as pharmaceutical or food composition, is claimed.
- No patents use *R. inulinivorans* or a combination of members of this genus for the treatment of IBD.

10.4. A possible biotechnological solution derived from this thesis.

As mentioned before, using several compositions for treating IBD as probiotics is common. In this thesis was observed that non-flagellated *R. inulinivorans* could increase the colonization ability of this bacteria, then this improvement in colonization may produce a better candidate for the treatment of IBD than the wild-type strain. It is also possible that other members of the *Roseburia* genus present the same behavior if flagellum is lost, then following the same strategy used for this thesis, non-flagellated bacteria from *Roseburia* members can be generated. This thesis did not study possible treatments for IBD, and more analysis should be done for this purpose, but if the experiments show promising results, some products and methods that can be claimed in a patent are:

1. A bacterial strain for use in the treatment of IBD, wherein the strain is a non-flagellated bacterium from the *Roseburia* genus.
2. The bacterial strain for use according to claim 1, the bacterial strain is a *Roseburia inulinivorans-derived* mutant (Include code of depository number).
3. A composition that includes the bacterium in claims 1 and 2 for treating of IBD at [X] CFU/mL and [other components].
4. A method for the treatment of IBD that includes the composition of claim 3.

This is narrow protection, and for sufficiency of the disclosure requirement, the strain must be deposited at a depository of bacterial strains. However, as *R. inulinivorans* mutants were sequenced and genes that generate this phenotype are known, it is possible to make narrower claims:

5. A bacterial strain for use in the treatment of IBD, wherein the strain is from the *Roseburia* genus and has one or more mutations in the basal body of flagellum.
6. The bacterial strain for use according to claim 5, is a non-flagellated strain belonging to *Roseburia inulinivorans*.
7. The bacterial strain for use according to claim 6, the bacterial strain is *Roseburia inulinivorans* with mutations in FliM and/or FliP.
8. A composition that includes the bacterium in claims 5, 6, and 7 for treating IBD at [X] CFU/mL and [other components].
9. A method for the treatment of IBD that includes the composition of claim 8.

As in this thesis, we work only with *R. inulinivorans,* claims probably could be accepted for this bacterium specie, but mutants in other members of the *Roseburia* genus should not

be protected. Thus, it is necessary to study the effect of the mutations in other species of this genus to protect them and generate protection around bacterial mixtures.

10.5. Novelty.

It has not been used mutant in flagellum from *R. inulinivorans* or other members of this genus for treating any disease, including IBD or related gastrointestinal diseases. Then, the novelty requirement is fulfilled.

10.6. Inventive step and non-obviousness.

Since the flagellum is an important colonization factor for many bacteria, mutations that generate the flagellum loss may not necessarily be associated with improving the colonization process. Moreover, an improvement in the colonization process should not necessarily generate an improvement in the treatment. Then, if studies show an increase in colonization and the effectiveness of the treatment for this disease, inventive steps and non-obviousness will be fulfilled.

11. CONCLUSIONS.

11.1. Conclusions of specific objective 1.

It was possible to generate non-motile mutants of *R. inulinivorans* using chemical mutagenesis using EMS accoupled with a high throughput screening strategy. This screening includes a repetitive passage in semi-solid plates that only allows, in analyzed strains, to isolate non-flagellated strains. Even when this non-motile phenotype could also be produced by non-functional flagellum, it also was possible to obtain revertant strains from non-flagellated mutants that involve multiple passages in semi-solid plates with a minimum of 50 generations.

11.2. Conclusions of specific objective 2.

Non-motile strains analyzed were non-flagellated strains exclusively. WGS demonstrated that *flhB*, *fliM*, and *fliP* were mutated in non-flagellated strains, but only *fliM* and *fliP* were genetically reverted. Then, it was possible to confirm that *fliM* and *fliP* are functional in *R. inulinivorans* and are required or flagellum assembly, while *flhB* mutation has to be studied more to determine the functionality and requirement for flagellum assembly in this bacterium. Revertant strains generate higher motility, which could be related to mutations in structural proteins, such as *fliF* and *fliM* observed in Rev12. Also, a mutation in CheY, could explain the higher motility in others revertants (Rev6 and Rev8), but more studies have to be done to determine the actual effect of these mutations on the higher-motility phenotype.

11.3. Conclusions of specific objective 3.

The flagellum is not required for adherence to epithelial cells *in vitro*, indeed flagellum loss in *R. inulinivorans* allows a higher adherence to this *in vitro* model. Also, this adherence could be improved when flagellin-antibodies interact with this bacterium. This phenotype is related to biofilm formation because flagellum loss increases biofilm formation *in vitro*. Thus, both epithelial cell adherence and biofilm formation could be related mechanisms. Conversely, the flagellum participates in adherence to mucin *in vitro*. Then, flagellum loss causes a decrease in this phenotype which also occurs when bacteria interact with anti-flagellin antibodies.

In vivo experiments did not show significant differences in gut colonization, but this could be a problem of the model used and the limitations of this study. Therefore, it is necessary to generate a better model to study colonization in *R. inulinivorans*. Having said this, our study shows an interesting tendency that makes it relevant to study if flagellum is required or could cause an impairment in the gut colonization of this bacteria.

11.4. Conclusion of biotechnological projections.

The relevance of *Roseburia* in the gut has made many studies and patents include members of this genus. Then, determining the colonization mechanisms on these species is relevant and could have a high impact in the development of microbes-based treatment for several diseases, principally associated with intestinal inflammation or disturbances.

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13. ANNEXES

13.1. Supplementary tables.

Table S1. Mutations per mutant strains compared to wild-type strain.

Strain	Mutation type	Annotation	Description
Mut6	Deletion (DEL)	Δ123 bp INTERGENIC (–/–)	–/–
Mut6	Deletion (DEL)	Δ105 bp	Unknown
Mut6	Deletion (DEL)	Δ123 bp	Unknown
Mut6	Multiple-base substitution (SUB)	INTERGENIC (–/+177)	Hypothetical protein
Mut6	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut6	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut6	Single-base substitution (SNP)	A87V (G<u>C</u>C→G<u>T</u>C)	Hypothetical protein
Mut6	Single-base substitution (SNP)	Y389Y (TA<u>C</u>→TA<u>T</u>)	Hypothetical protein
Mut6	Single-base substitution (SNP)	Q126* (<u>C</u>AG→<u>T</u>AG)	Flagellar biosynthesis protein flip
Mut6	Single-base substitution (SNP)	S135N (A<u>G</u>C→A<u>A</u>C)	Ribonuclease HII (EC 3.1.26.4)
Mut6	Single-base substitution (SNP)	P18R (C<u>C</u>T→C<u>G</u>T)	Hypothetical protein
Mut6	Single-base substitution (SNP)	G277D (G<u>G</u>C→G<u>A</u>C)	Eric-type fluoride/proton exchange protein

Mut6	Single-base substitution (SNP)	INTERGENIC (–/+159)	Hypothetical protein
Mut8	Deletion (DEL)	Δ113 bp INTERGENIC (–/–)	–/–
Mut8	Deletion (DEL)	Δ123 b INTERGENIC (–/–)	–/–
Mut8	Deletion (DEL)	Δ123 b INTERGENIC (–/–)	–/–
Mut8	Deletion (DEL)	Δ123 b	Unknown
Mut8	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut8	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut8	Single-base insertion on repeat base	INTERGENIC (+151/-65)	LSU ribosomal protein l19p/hypothetical protein
Mut8	Single-base substitution (SNP)	A87V (G<u>C</u>C→G<u>T</u>C)	Hypothetical protein
Mut8	Single-base substitution (SNP)	Y389Y (TA<u>C</u>→TA<u>T</u>)	Hypothetical protein
Mut8	Single-base substitution (SNP)	F315F (TT<u>C</u>→TT<u>T</u>)	Hypothetical protein
Mut8	Single-base substitution (SNP)	INTERGENIC (+175/–)	Hypothetical protein/–
Mut8	Single-base substitution (SNP)	S135N (A<u>G</u>C→A<u>A</u>C)	Ribonuclease HII (EC 3.1.26.4)
Mut8	Single-base substitution (SNP)	G277D (G<u>G</u>C→G<u>A</u>C)	Eric-type fluoride/proton exchange protein
Mut8	Single-base substitution (SNP)	INTERGENIC (-123/–)	Methyl-accepting chemotaxis sensor/transducer protein/–

Mut8	Single-base substitution (SNP)	Q126* (CAG→TAG)	Flagellar biosynthesis protein flip
Mut12	Deletion (DEL)	Δ105 bp	Unknown
Mut12	Deletion (DEL)	Δ105 bp	Unknown
Mut12	Deletion (DEL)	Δ24 bp	Unknown
Mut12	Multiple-base substitution (SUB)	INTERGENIC (–/+177)	–/Hypothetical protein
Mut12	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut12	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut12	Single-base substitution (SNP)	Q447* (CAG→TAG)	ATP-dependent protease La (EC 3.4.21.53) Type I
Mut12	Single-base substitution (SNP)	V249I (GTT→ATT)	Cysteine desulfurase (EC 2.8.1.7), associated with trna 4-thiouridine synthase
Mut12	Single-base substitution (SNP)	INTERGENIC (+175/–)	Hypothetical protein/–
Mut12	Single-base substitution (SNP)	INTERGENIC (–/+134)	–/Hypothetical protein
Mut12	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–
Mut12	Single-base substitution (SNP)	T364I (ACA→ATA)	Sodium-dependent phosphate transporter
Mut12	Single-base substitution (SNP)	D36D (GAT→GAC)	Hypothetical protein
Mut12	Single-base substitution (SNP)	INTERGENIC (-123/–)	Methyl-accepting chemotaxis sensor/transducer protein/–

Mut12	Single-base substitution (SNP)	W167* (TGG→TGA)	Flagellar motor switch protein flim
Mut12	Single-base substitution (SNP)	T226I (ACC→ATC)	Oxygen-independent coproporphyrinogen-III oxidase-like protein yggw
Mut14	Deletion (DEL)	Δ105 bp CODING (1-105/105 NT)	Hypothetical protein
Mut14	Deletion (DEL)	Δ136 bp INTERGENIC (–/–)	–/–
Mut14	Deletion (DEL)	Δ13 bp CODING (646-658/1749 NT)	Hypothetical protein
Mut14	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut14	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut14	Single-base substitution (SNP)	A87V (GCC→GTC)	Hypothetical protein
Mut14	Single-base substitution (SNP)	Y389Y (TAC→TAT)	Hypothetical protein
Mut14	Single-base substitution (SNP)	T253N (ACT→AAT)	Hypothetical protein
Mut14	Single-base substitution (SNP)	S135N (AGC→AAC)	Ribonuclease HII (EC 3.1.26.4)
Mut14	Single-base substitution (SNP)	G277D (GGC→GAC)	Eric-type fluoride/proton exchange protein
Mut14	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–

Mut14	Single-base substitution (SNP)	Q126* (CAG→TAG)	Flagellar biosynthesis protein flip
Mut14	Single-base substitution (SNP)	P19P (CCG→CCA)	Hypothetical protein
Mut14	Single-base substitution (SNP)	E213D (GAA→GAT)	Undecaprenyl diphosphate synthase (EC 2.5.1.31)
Mut15	Deletion (DEL)	Δ123 bp	Unknown
Mut15	Deletion (DEL)	Δ105 bp CODING (1-105/105 NT)	Hypothetical protein
Mut15	Deletion (DEL)	Δ123 bp INTERGENIC (–/–)	–/–
Mut15	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut15	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut15	Single-base substitution (SNP)	A277T (GCA→ACA)	TPP-requiring enzyme co-localized with putative O-antigen rfb gene cluster
Mut15	Single-base substitution (SNP)	INTERGENIC (–/+134)	–/Hypothetical protein
Mut15	Single-base substitution (SNP)	G56E (GGA→GAA)	Possible microcin immunity protein
Mut15	Single-base substitution (SNP)	T364I (ACA→ATA)	Sodium-dependent phosphate transporter
Mut15	Single-base substitution (SNP)	D36D (GAT→GAC)	Hypothetical protein
Mut15	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–

Mut15	Single-base substitution (SNP)	W17* (TGG→TAG)	Flagellar biosynthesis protein flhb
Mut17	Deletion (DEL)	Δ123 bp	Unknown
Mut17	Deletion (DEL)	Δ113 bp INTERGENIC (–/–)	–/–
Mut17	Deletion (DEL)	Δ130 bp	*[Unknown]*
Mut17	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut17	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut17	Single-base substitution (SNP)	INTERGENIC (–/+150)	–/Hypothetical protein
Mut17	Single-base substitution (SNP)	A277T (GCA→ACA)	TPP-requiring enzyme co-localized with putative O-antigen rfb gene cluster
Mut17	Single-base substitution (SNP)	INTERGENIC (–/+134)	–/Hypothetical protein
Mut17	Single-base substitution (SNP)	G56E (GGA→GAA)	Possible microcin immunity protein
Mut17	Single-base substitution (SNP)	T364I (ACA→ATA)	Sodium-dependent phosphate transporter
Mut17	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–
Mut17	Single-base substitution (SNP)	D36D (GAT→GAC)	Hypothetical protein
Mut17	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–
Mut17	Single-base substitution (SNP)	W17* (TGG→TAG)	Flagellar biosynthesis protein flhb

Mut19	Deletion (DEL)	Δ123 bp	Unknown
Mut19	Deletion (DEL)	Δ105 bp CODING (1-105/105 NT)	Hypothetical protein
Mut19	Deletion (DEL)	Δ105 bp INTERGENIC (–/–)	–/–
Mut19	Deletion (DEL)	INTERGENIC (–/–)	–/–
Mut19	Single-base deletion on repeat base	INTERGENIC (+151/-65)	LSU ribosomal protein l19p/hypothetical protein
Mut19	Single-base deletion on repeat base	CODING (35/1494 NT)	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
Mut19	Single-base deletion on repeat base	CODING (13/960 NT)	Spore germination protein
Mut19	Single-base substitution (SNP)	INTERGENIC (-78/-259)	Flagellar hook-associated protein flid/LSU ribosomal protein L33p @ LSU ribosomal protein l33p, zinc-dependent
Mut19	Single-base substitution (SNP)	D191D (GA<u>C</u>→GA<u>T</u>)	Mobile element protein
Mut19	Single-base substitution (SNP)	G635D (G<u>G</u>C→G<u>A</u>C)	Beta-galactosidase (EC 3.2.1.23)
Mut19	Single-base substitution (SNP)	D311N (<u>G</u>AT→<u>A</u>AT)	Uncharacterized protease yrro
Mut19	Single-base substitution (SNP)	F315F (TT<u>C</u>→TT<u>T</u>)	Hypothetical protein
Mut19	Single-base substitution (SNP)	G85E (G<u>G</u>G→G<u>A</u>G)	Hypothetical protein
Mut19	Single-base substitution (SNP)	W270* (TG<u>G</u>→TG<u>A</u>)	Flagellar biosynthesis protein flip
Mut19	Single-base substitution (SNP)	T364I (A<u>C</u>A→A<u>T</u>A)	Sodium-dependent phosphate transporter

Mut19	Single-base substitution (SNP)	D36D (GA<u>T</u>→GA<u>C</u>)	Hypothetical protein
Mut19	Single-base substitution (SNP)	INTERGENIC (–/–)	–/–
Mut19	Single-base substitution (SNP)	R262H (C<u>G</u>C→C<u>A</u>C)	Ribonuclease Y
Mut19	Single-base substitution (SNP)	R240R (CG<u>C</u>→CG<u>T</u>)	Hypothetical protein

Table S2. Doubling time at 6, 9, and 12 h for wild type and mutant strains.

Strain	**t_d at 6 h**		**t_d at 9 h**		**t_d at 12 h**	
	t_d	SD	t_d	SD	t_d	SD
Wild type	1.03	0.05	2.41	1.34	1.49	0.06
Mut1	1.34	0.20	1.60	0.09	1.56	0.01
Mut2	1.28	0.01	1.57	0.05	1.60	0.07
Mut3	1.18	0.06	1.89	0.50	2.16	0.90
Mut4	12.97	1.68	3.28	0.30	4.37	0.27
Mut5	1.18	0.01	1.83	0.10	1.65	0.11
Mut6	1.09	0.11	1.49	0.03	1.54	0.12
Mut7	1.16	0.23	1.58	0.04	1.55	0.08
Mut8	1.16	0.12	1.54	0.20	1.53	0.17
Mut9	23.15	23.08	24.90	8.25	35.39	2.00
Mut10	1.08	0.04	1.36	0.04	1.48	0.03
Mut11	1.17	0.04	1.54	0.05	1.54	0.12
Mut12	1.10	0.04	1.41	0.00	1.45	0.09
Mut13	8.21	4.11	29.83	23.88	26.50	1.04
Mut14	1.11	0.02	1.55	0.02	1.50	0.05
Mut15	1.17	0.02	1.66	0.06	1.59	0.01

Mut16	1.08	0.12	1.46	0.08	1.54	0.03
Mut17	1.25	0.09	3.14	2.10	1.57	0.04
Mut18	1.14	0.11	1.60	0.17	1.56	0.16
Mut19	1.20	0.03	1.43	0.06	1.50	0.02

Table S3. Mutations in revertant strains compared to the wild-type strain.

Strain	Mutation type	Annotation	Description
Rev6	Single-base substitution (SNP)	D99E (GAC→GAA)	Chemotaxis regulator transmits chemoreceptor signals to flagellar motor components CheY
Rev6	Multiple-base substitution (SUB)	coding (376-377/849 nt)	Flagellar biosynthesis protein FliP
Rev6	Single-base substitution (SNP)	N383D (AAT→GAT)	hypothetical protein
Rev6	Single-base substitution (SNP)	G255E (GGG→GAG)	hypothetical protein
Rev6	Single-base substitution (SNP)	S243* (TCG→TAG)	Sodium-dependent phosphate transporter
Rev8	Single-base substitution (SNP)	A113E (GCA→GAA)	Chemotaxis regulator - transmits chemoreceptor signals to flagellar motor components CheY
Rev8	Single-base substitution (SNP)	Q126Y (CAG→TAT)	Flagellar biosynthesis protein FliP
Rev8	Single-base substitution (SNP)	Q126Y (CAG→TAT)	Flagellar biosynthesis protein FliP
Rev8	Single-base substitution (SNP)	Y19C (TAC→TGC)	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)

Rev12	Single-base substitution (SNP)	R42C (CGT→TGT)	Flagellar motor switch protein FliM
Rev12	Single-base substitution (SNP)	W528* (TGG→TGA)	Flagellar M-ring protein FliF
Rev12	Deletion (DEL)	Δ9 bp coding (2726 2734/3630 nt)	DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)
Rev12	Single-base substitution (SNP)	A217V (GCG→GTG)	ABC transporter, substrate-binding protein
Rev12	Single-base substitution (SNP)	R140H (CGC→CAC)	Carbonic anhydrase, gamma class (EC 4.2.1.1)
Rev12	Single-base substitution (SNP)	P414P (CCC→CCT)	Hypothetical protein
Rev12	Single-base substitution (SNP)	S547F (TCC→TTC)	hypothetical protein
Rev12	Single-base substitution (SNP)	Y2293Y (TAC→TAT)	hypothetical protein
Rev12	Single-base substitution (SNP)	I177I (ATC→ATT)	NAD(P)H-hydrate epimerase (EC 5.1.99.6) / ADP-dependent (S)-NAD(P)H-hydrate dehydratase (EC 4.2.1.136)
Rev12	Single-base substitution (SNP)	D50Y (GAT→TAT)	NADP-dependent malic enzyme (EC 1.1.1.40)
Rev12	Single-base substitution (SNP)	S101T (TCG→ACG)	Peptide chain release factor 2
Rev19	Single-base substitution (SNP)	W270C (TGG→TGC)	Flagellar biosynthesis protein FliP
Rev19	Deletion (DEL)	Δ35 bp Coding (990-1024/1077 nt)	hypothetical protein
Rev19	Large deletion	Δ3,587 bp	Unknown
Rev19	Single-base substitution (SNP)	P552T (CCA→ACA)	hypothetical protein
Rev19	Single-base substitution (SNP)	L36L (TTG→TTA)	hypothetical protein

Rev19	Single-base substitution (SNP)	noncoding (28/73 nt)	tRNA-Ala-TGC

Table S4. Comparative table of gene mutations presence in S6, S8, and S12 compared to wild-type strain.

Description [Mutation type = Result]	Mut6	Rev6	Mut8	Rev8	Mut12	Rev12
ABC transporter, substrate-binding protein (cluster 15, trp) [Single-base substitution = Nonsynonymous mutation]						
ATP-dependent protease La (EC 3.4.21.53) Type I [Single-base substitution = Nonsense mutation]						
Carbonic anhydrase, gamma class (EC 4.2.1.1) [Single-base substitution = Nonsense mutation]						
Chemotaxis regulator - transmits chemoreceptor signals to flagellar motor components CheY ABC transporter, substrate-binding protein (cluster 15, trp) [Single-base substitution = Nonsynonymous mutation]						
Cysteine desulfurase (EC 2.8.1.7), associated with tRNA 4-thiouridine synthase [Single-base substitution = Nonsynonymous mutation]						
DNA-directed RNA polymerase beta subunit (EC 2.7.7.6) [Single-base substitution = Nonsynonymous mutation]						
DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6) [Multiple-base deletion = Frameshift mutation]						
EriC-type fluoride/proton exchange protein [Single-base substitution = Nonsynonymous mutation]						

Flagellar biosynthesis protein FliP [Single-base substitution = Nonsense mutation]						
Flagellar biosynthesis protein FliP [Multiple-base substitution = Frameshift mutation]						
Flagellar biosynthesis protein FliP [Two single-base substitution = Nonsynonymous mutations]						
Flagellar motor switch protein FliM [Single-base substitution = Nonsense mutation]						
Flagellar motor switch protein FliM [Single-base substitution = Nonsynonymous mutation]						
Flagellar M-ring protein FliF [Single-base substitution = Nonsense mutation]						
NAD(P)H-hydrate epimerase (EC 5.1.99.6) / ADP-dependent (S)-NAD(P)H-hydrate dehydratase (EC 4.2.1.136) [Single-base substitution = Nonsynonymous mutation]						
NADP-dependent malic enzyme (EC 1.1.1.40) [Single-base substitution = Nonsynonymous mutation]						
Oxygen-independent coproporphyrinogen-III oxidase-like protein YggW [Single-base substitution = Nonsynonymous mutation]						
Peptide chain release factor 2 [Single-base substitution = Nonsynonymous mutation]						
Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-) [Single-base deletion = Frameshift mutation]						
Ribonuclease HII (EC 3.1.26.4) [Single-base substitution = Nonsynonymous mutation]						

Sodium-dependent phosphate transporter [Single-base substitution = Nonsense mutation]						
Sodium-dependent phosphate transporter [Single-base substitution = Nonsynonymous mutation]						
Spore germination protein [Single-base deletion = Frameshift mutation]						

* Green colored cells indicated presence of defined mutations from the first column.

* No intergenic, hypothetical or unknown proteins mutations were included.

13.2. Scientific articles published during the doctorate.

- Díaz-Yáñez, F., Álvarez, R., Calderón, I. L., Fuentes, J. A., & Gil, F. (2020). CdsH contributes to the replication of S*almonella* typhimurium inside epithelial cells in a cysteine-supplemented medium. *Microorganisms*, *8*(12), 2019.
- Díaz-Yáñez, F., Castro-Córdova, P., Muñoz-Miralles, J., Gil, F., & Paredes-Sabja, D. (2020). Effect of antibiotic to induce *Clostridioides difficile*-susceptibility and infectious strain in a mouse model of *Clostridioides difficile* infection and recurrence. *Anaerobe*, *62*, 102149.
- Álvarez, R., Ortega-Fuentes, C., Queraltó, C., Inostroza, O., Díaz-Yáñez, F., González, R., ... & Gil, F. (2020). Evaluation of functionality of type II toxin-antitoxin systems of *Clostridioides difficile* R20291. *Microbiological Research*, *239*, 126539.
- Queraltó, C., Álvarez, R., Ortega, C., Díaz-Yáñez, F., Paredes-Sabja, D. & Gil, F. (2022). Role and regulation of Clp proteases: a target against Gram-positive bacteria. *Bacteria*. Accepted manuscript.