Insights into c-di-GMP signaling and the PGA exopolysaccharide biological functions using *Salmonella* as a model organism

Memoria presentada por

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INFORMAN:

Que la presente memoria de Tesis Doctoral "**Insights into c-di-GMP signaling and the PGA exopolysaccharide biological functions using *Salmonella* as a model organism**" elaborada por Doña **MAITE ECHEVERZ** ha sido realizada bajo su dirección y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona, a 17 de Febrero de 2017

Fdo. Íñigo Lasa Uzcudun

Fdo. Cristina Solano Goñi

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RESUMEN

RESUMEN

Salmonella es un patógeno alimentario de gran relevancia clínica capaz de adherirse a superficies y formar comunidades bacterianas embebidas en una matriz que ellas mismas producen denominadas biofilms. Esta matriz confiere a las bacterias protección frente a agentes externos, aumentando su tolerancia frente a condiciones ambientales adversas, agentes antimicrobianos o el sistema inmune del hospedador. Existe una vía de señalización, mediada por el dinucleótido cíclico, c-di-GMP, que controla en muchas especies bacterianas la síntesis de diversos componentes de la matriz del biofilm, de manera que concentraciones elevadas de este nucleótido activan la producción de la matriz y por lo tanto del biofilm. La formación de biofilms en explotaciones agropecuarias y lugares donde se procesan alimentos es una fuente potencial de contaminación y de transmisión de este patógeno. Diversas medidas de higiene y seguimiento han sido implementadas por las autoridades para el control de esta bacteria; sin embargo alrededor de 93 millones de personas en todo el mundo sufren salmonelosis cada año. Por ello, la búsqueda de medidas alternativas de control, basadas en la vacunación animal, así como el estudio de los mecanismos de patogenicidad y formación de biofilm de *Salmonella* han sido el objeto de este trabajo.

La primera parte de esta tesis se ha dedicado a la evaluación de una cepa atenuada de *Salmonella* para su posible uso como cepa vacunal. En primer lugar, se analizó la implicación del sistema de señalización mediado por c-di-GMP en la virulencia de *Salmonella* mediante la comparación, en diversos modelos de infección en ratón, de la virulencia de una cepa salvaje y de una cepa mutante múltiple en todos los genes responsables de la síntesis de c-di-GMP, y por lo tanto incapaz de sintetizar c-di-GMP. Este mutante presentó una atenuación moderada, indicando que el sistema de señalización del c-di-GMP es necesario durante la infección. La mutación

adicional, en este mutante, del gen *rpoS* dio lugar a una cepa, denominada ΔXIII, muy atenuada y capaz de inducir una respuesta inmune protectora frente a un desafío con una cepa virulenta de *S.* Typhimurium. Además, esta cepa vacunal presenta una serie de características que indican que no sería capaz de sobrevivir en el ambiente y que, por otra parte, permitirían la diferenciación de animales vacunados y no vacunados. Por todo ello, se propone el uso de la cepa ΔXIII como una cepa vacunal viva y atenuada para la protección frente a *Salmonella* de los animales implicados en la transmisión de esta bacteria.

Posteriormente y recogido en el capítulo dos, hemos estudiado la implicación del receptor eucariota del c-di-GMP, denominado STING (STimulator of INterferon Genes), durante la infección por *Salmonella*. Recientemente se ha descrito que STING es una proteína de la célula huésped capaz de unir c-di-GMP y generar una respuesta inmune a través de la producción de interferón. Sin embargo se desconoce si en el caso de *Salmonella*, tal y como ocurre en otras bacterias, esta producción de IFN puede suponer una estrategia de la bacteria para modular la respuesta inmune del hospedador, o por el contrario la activación de STING representa un mecanismo del huésped de protección frente a la infección. Para llevar a cabo este estudio, hemos utilizado una cepa salvaje y el mutante múltiple incapaz de sintetizar c-di-GMP y hemos realizado infecciones *in vitro* e *in vivo* utilizando ratones salvajes y mutantes en STING. Nuestros resultados indican que la infección de macrófagos con *Salmonella* da lugar a la producción de IFN-β mediada por STING, independientemente de la presencia de c-di-GMP. Por otra parte, tanto la cepa salvaje de *Salmonella* como el mutante en c-di-GMP son capaces de infectar de igual manera ratones salvajes y deficientes en STING, lo cual demuestra que ni el reconocimiento del c-di-GMP a través de STING ni cualquier otro mecanismo de activación de STING juegan un papel

relevante en el resultado de la infección por *Salmonella*.

Por último, en el capítulo tres, hemos estudiado las razones por las cuales *Salmonella* no presenta en su genoma los genes necesarios para la síntesis del exopolisacárido PGA (poly-β-1,6-*N*-acetyl-D-glucosamine). La matriz del biofilm de *Salmonella* está formada fundamentalmente por el exopolisacárido celulosa; sin embargo otras enterobacterias como *Escherichia coli* además de sintetizar celulosa, recurren también al exopolisacarido PGA para la formación del biofilm. Teniendo en cuenta que *Salmonella* y *E. coli* se encuentran filogenéticamente muy relacionadas pero sus ciclos de vida son muy diferentes, nos planteamos que tal vez la ausencia de PGA supuso un evento evolutivo de diversificación entre estas especies. El análisis filogenético comparativo de genomas de enterobacterias sugiere que *Salmonella* perdió los genes implicados en la síntesis de PGA después de la diversificación de los géneros *Salmonella* y *Citrobacter* y antes de la diversificación de las distintas especies de *Salmonella*. Mediante la expresión heteróloga del polisacárido PGA de *Escherichia coli* en *Salmonella* hemos podido determinar que al igual que ocurre en *E. coli*, la síntesis de PGA es dependiente de c-di-GMP. Hemos observado que la producción de PGA da lugar a la formación de un biofilm muy diferente a nivel estructural de la celulosa que no proporciona una ventaja adicional para la supervivencia de la bacteria frente a diferentes estreses ambientales. Por otra parte, la producción de PGA resulta muy perjudicial para la supervivencia de *Salmonella* dentro del huésped, ya que aumenta la sensibilidad a las sales biliares y especies reactivas de oxigeno, comprometiendo su capacidad de supervivencia en macrófagos y la colonización de órganos incluyendo la vesícula biliar. Por lo tanto, nuestros resultados indican que el PGA es un factor antivirulencia en *Salmonella*, cuya pérdida supuso una estrategia evolutiva necesaria para garantizar la colonización y supervivencia en el huésped.

Secciones de esta Tesis Doctoral han sido publicadas en:

- **Echeverz M, Latasa C, García B, Gil C, García-Ona E, Burgui S, Casares N, Hervás-Stubbs, Lasarte J. J, Lasa I, Solano C**. 2016. Evaluation of a *Salmonella* strain lacking the secondary messenger c-di-GMP and RpoS as a live oral vaccine. PLoS ONE, **11**: e0161216.

Otras publicaciones relacionadas con el presente trabajo y en las que la doctoranda es coautora:

1. **Solano C**, **Echeverz M**, **Lasa I**. 2014. Biofilm dispersion and quorum sensing. Curr Opin in Microbiol **18**:96–104.
2. **Zorraquino V**, **García B**, **Latasa C**, **Echeverz M**, **Toledo-Arana A**, **Valle J**, **Lasa I**, **Solano C**. 2013. Coordinated cyclic-di-GMP repression of *Salmonella* motility through YcgR and cellulose. J Bacteriol **195**:417–428.
3. **Latasa C**, **García B**, **Echeverz M**, **Toledo-Arana A**, **Valle J**, **Campoy S**, **García-del Portillo F**, **Solano C**, **Lasa I**. 2012. *Salmonella* biofilm development depends on the phosphorylation status of RcsB. J Bacteriol **194**:3708–3722.

SUMMARY

SUMMARY

Salmonella is a foodborne pathogen of clinical relevance able to adhere to surfaces and form biofilms that are defined as bacterial communities embedded in a self-produced matrix. This matrix confers upon bacteria protection against external agents, increasing their resistance to adverse environmental conditions, antimicrobial agents and the host immune system. There is a signaling pathway mediated by the cyclic dinucleotide c-di-GMP, which controls, in many bacterial species, the synthesis of various components of the biofilm matrix, so that high concentrations of this nucleotide activate matrix production and biofilm formation. Biofilm formation in farms and food processing sites is a potential source of contamination and transmission of this pathogen. In spite of hygiene and control measures implemented by the authorities for the control of this bacterium, around 93 million people suffer from salmonellosis every year, worldwide. Therefore, the search for alternative control measures, based on animal vaccination, as well as the study of *Salmonella* pathogenicity mechanisms and the biofilm formation process have been the subject of this work.

The first part of this thesis has been focused on the evaluation of a *Salmonella* strain as a putative vaccine candidate. First, the implication of the c-di-GMP-mediated signaling system in *Salmonella* virulence was analyzed by comparing the virulence of a wild-type strain with that of a multiple mutant strain in all the genes responsible for c-di-GMP synthesis, and therefore unable to synthesize c-di-GMP. This mutant showed a slight but significant attenuation in virulence, indicating that the c-di-GMP signaling system is required during infection. An additional deletion in this mutant of the *rpoS* gene resulted in a highly attenuated strain, named ΔXIII, capable of inducing a protective immune response against a challenge with a virulent *S.* Typhimurium strain. In addition, this vaccine strain showed

several features that indicate that it would not be able to survive in the environment and that, on the other hand, would allow the differentiation between vaccinated and unvaccinated animals. Therefore, we propose the use of ΔXIII strain as a live and attenuated vaccine against *Salmonella* for the protection of animals involved in the transmission of this pathogen.

Then, chapter two is dedicated to analyze the involvement of the c-di-GMP eukaryotic receptor STING (STimulator of INterferon Genes) in *Salmonella* infection. It has recently been described that STING is a host cell protein capable of binding c-di-GMP and generating an immune response through the production of interferon. However, it is not known whether, in the case of *Salmonella*, stimulation of IFN production might be a bacterial strategy to modulate the host immune response, as it happens with other bacteria, or on the contrary, STING activation represents a host mechanism for protection against infection. To carry out this study, we used a wild type strain and the multiple mutant unable to synthesize c-di-GMP to perform *in vitro* and *in vivo* infections, using wild type and STING deficient mice. Our results indicate that macrophages infection with *Salmonella* results in the production of IFN-β mediated by STING, regardless of the presence of c-di-GMP. On the other hand, both the wild-type and the c-di-GMP mutant strains are equally able to infect wild-type and STING deficient mice, showing that neither recognition of c-di-GMP via STING nor a different mechanism of STING activation play a relevant role in the outcome of a *Salmonella* infection.

Finally, in chapter three, we have studied the reasons why *Salmonella* does not harbor the genes necessary for the synthesis of the PGA (poly-β-1,6-N-acetyl-D-glucosamine) exopolysaccharide. The *Salmonella* biofilm matrix is mainly formed by cellulose. However, other bacteria from the *Enterobacteriaceae* family such as *Escherichia coli*, synthesize not only cellulose, but also PGA for biofilm formation. Taking into

account that *Salmonella* and *E. coli* are phylogenetically very related and that their life cycles are very different, we considered that perhaps, absence of PGA in *Salmonella* was an evolutionary event of diversification amongst these species. Comparative phylogenetic analyses of *Enterobacteriaceae* genomes suggest that *Salmonella* lost the genes involved in PGA synthesis after the diversification of the *Salmonella* and *Citrobacter* clades and previous to the diversification of the currently sequenced *Salmonella* strains. Heterologous expression of the *Escherichia coli* PGA polysaccharide in *Salmonella* allowed us to determine that, as it happens in *E. coli*, PGA synthesis is dependent on c-di-GMP. PGA production in *Salmonella* leads to the formation of a biofilm that is very different at a structural level from the biofilm formed by cellulose and that does not provide any additional advantage for bacterial survival against different environmental stresses. On the other hand, PGA production is very detrimental to *Salmonella* survival inside the host, since it increases the sensitivity to bile salts and reactive oxygen species, compromising *Salmonella* survival inside macrophages and also organ colonization, including that of the gallbladder. Therefore, our results indicate that PGA is an antivirulence factor in *Salmonella*, whose loss may have been an evolutionary strategy to guarantee colonization and survival inside the host.

Sections of this Doctoral Thesis have been published in:

- **Echeverz M, Latasa C, García B, Gil C, García-Ona E, Burgui S, Casares N, Hervás-Stubbs, Lasarte J. J, Lasa I, Solano C**. 2016. Evaluation of a *Salmonella* strain lacking the secondary messenger c-di-GMP and RpoS as a live oral vaccine. PLoS ONE, **11**: e0161216.

Other publications in which the candidate has participated as a co-author and that are related with this thesis:

1. **Solano C**, **Echeverz M**, **Lasa I**. 2014. Biofilm dispersion and quorum sensing. Curr Opin in Microbiol **18**:96–104.
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INTRODUCTION

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The genus *Salmonella*

Taxonomy of the genus *Salmonella*

The genus *Salmonella* belongs to the Enterobacteriaceae family. This genus contains flagellated, non-sporulated, facultative anaerobic, gram-negative bacilli. Based on DNA-DNA hybridization assays, the genus has been divided into two species, *S. enterica* and *S. bongori* (Reeves *et al.*, 1989; Euzeby, 1999; Shelobolina *et al.*, 2004; Tindall, 2005). *Salmonella enterica* is further divided into six subspecies named as:

S. *enterica* subsp. *enterica* (I)
S. *enterica* subsp. *salamae* (II)
S. *enterica* subsp. *arizonae* (IIIa)
S. *enterica* subsp. *diarizonae* (IIIb)
S. *enterica* subsp. *houtenae* (IV)
S. *enterica* subsp. *indica* (VI)

These subspecies include more than 2500 different serovars that are classified based on the serologic identification of O (somatic) and H (flagellar) antigens and are listed in a document called the Kauffmann–White scheme (Grimont and François-Xavier, 2007). Whereas *S. bongori* and *S. enterica* subspecies II–IV and VI are isolated predominantly from poikilothermic vertebrate species, the host range of *S. enterica* subspecies I serovars includes homeothermic animals (i.e., mammals and birds). Importantly, approximately 99% of human clinical isolates belong to *S. enterica* subspecies I, and the vast majority of the 1,531 serovars of this subspecies are associated with gastroenteritis in immunocompetent individuals (Brenner *et al.*, 2000). From a clinical perspective, *Salmonella*

serovars are usually classified into two broad groups. Nontyphoidal *Salmonella* (NTS) serovars are considered to be generalist in terms of host range and have a low human invasiveness index, typically causing self-limiting enterocolitis in immunocompetent hosts. On the other hand, typhoidal serovars that encompass *S. enterica* serovars Typhi and Paratyphi, are human specific pathogens that cause enteric fever, a systemic illness due to dissemination to the lymph nodes, liver and spleen through the lymphatic system.

In this thesis, we have mainly worked with S. *enterica* subsp. *enterica* ser Enteritidis, which is hereafter abbreviated as *S.* Enteritidis.

Epidemiology

The primary habitat of *Salmonella* is the intestinal tract of humans and other animals (mammals, reptiles, birds and even insects), though it can also survive for long periods of time outside the host withstanding a variety of stresses associated with environmental fluctuations (Winfield and Groisman, 2003). This adaptability allows *Salmonella* to transit from the environment to the host during its life cycle (Winfield and Groisman, 2003).

Salmonella is one of the most common etiologic agents of foodborne diarrhoea and enteric fever in the world, mainly in non-developed countries. In humans, *Salmonella* infection usually results from the ingestion of water and food contaminated products, such as undercooked beef, pork, chicken, seafood or eggs (Pang *et al.*, 1995), although there is also evidence of *Salmonella* infections following the consumption of fresh fruits or vegetables that have been contaminated by infected fertilizers (Tauxe, 1997) and even of transmission between reptilian pets and their owners (Cain *et al.*, 2009).

In the European Union (EU), a total of 88,715 clinical cases of salmonellosis were reported in 2014. The European Food Safety Authority (EFSA) has estimated that the overall economic burden of human

salmonellosis could be as high as 3 billion euros a year (http://onlinelibrary.wiley.com/doi/10.2903/j.efsa.2015.4329/epdf). In Europe, the two most commonly detected serovars in the year 2014 were *S.* Typhimurium and *S.* Enteritidis, two ubiquitous serovars whose lack of specificity in host adaptation permits them to colonize a wide variety of organisms (Uzzau *et al.*, 2000), representing 44.4% and 17.4%, respectively, of all reported serovars in confirmed human cases. The reporting of human *S.* Enteritidis cases increased, whereas *S.* Typhimurium and other common serovars cases decreased in 2014 compared with 2103. As in previous years, *Salmonella* was most frequently detected in poultry meat, and less often in pig or bovine meat. The most important source of food-borne *Salmonella* outbreaks was, however, still eggs and egg products.

In 2003, the European Union (EU) set up an extended control programme for zoonoses, considering *Salmonella* as a priority (Regulation (EC) Nº 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents), (http://eur-lex.europa.eu/legal-content/ES/TXT/?uri=CELEX:32003R2160). The purpose of this regulation was to ensure that proper and effective measures were taken to detect and control *Salmonella* and other zoonotic agents at all relevant stages of production, processing and distribution, particularly at the level of primary production, including in feed. The final aim of these interventions was reducing *Salmonella* prevalence and consequently the risk of reaching the human food chain. Enhanced *Salmonella* control programmes in poultry were implemented in all EU Member States. Targets were set for the reduction of *Salmonella* in poultry flocks (e.g. laying hens, broilers, turkeys) and pigs. Restrictions were also imposed on the trade of products from infected flocks.

Salmonellosis in Spain, as throughout the EU, is one of the main food-borne zoonosis. In 2014, 6.643 human cases were reported in Spain, and as in other countries of the EU the main focus of contamination was egg consumption. Several control programs have been approved to control *Salmonella* incidence in poultry farms in order to avoid foodstuff contamination. Since January the 1^{rst}, 2009, eggs cannot be used for direct human consumption as table eggs unless they originate from a commercial flock of laying hens subjected to a national *Salmonella* control programme. Also, fresh eggs from flocks found positive for *S.* Typhimurium or *S.* Enteritidis must be heat treated before consumption (Regulation (EC) Nº 1237/200723). According to Regulation (EC) Nº 2160/2003 and Regulation (EC) N^{o} 200/2010.2, control programmes for breeding flocks aim to meet a reduction target of 1% or less of positive flocks for the following serovars; *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Virchow and *S.* Hadar. In 2014, the percentage of positive breeding flocks of these serovars in the framework of this normative was 0,5% in Spain, and the total amount of *Salmonella* incidence was 4,31% (Table 1). While these control programs have been successful for reducing *Salmonella* incidence in poultry farms, the amount of human cases is still high, suggesting that additional control programs (to reduce *Salmonellosis* human cases) are needed.

In the case of pigs, although related infections due to pig meat consumption are fewer than in the case of poultry derived products, the incidence of *Salmonella* in european pig farms is still very high. At the moment, control programmes aimed at reducing *Salmonella* contamination of pig derived products and prevalence in farms are not mandatory, and thus, spanish data regarding *Salmonella* in pigs from bacteriological monitoring programmes are unavailable in the 2014 EFSA report. Although there is still not a national control programme established in Spain, a number of studies have been performed through the last decade that show

the high *Salmonella* prevalence in finishing herds.

High *Salmonella* incidence is due, amongst other factors, to the increased consumption of processed food, which implies a consequent risk of cold chain rupture during food transport, and also to the high-productivity-focused model of animal breeding in which antibiotics are extensively used leading to the spread of antibiotic resistance (Shakespeare *et al.*, 2005) (Parry, 2004) (González-Zorn and Escudero, 2012). This scenario demands the search of new strategies to reduce contamination incidence based on measures related to feeding practices, vaccination and generic measures of hygiene and biosecurity.

Table 1. *Salmonella* prevalence in Spanish farms

Breeding flocks of *Gallus gallus*	4,31 %
Laying hen flocks of *Gallus gallus*	7,67 %
Broiler flocks of *Gallus gallus*	3,63 %
Breeding turkeys flocks	9,38 %
Flattering turkeys flocks	17,5 %

Pathogenesis

Salmonella disease manifestation depends on both, host susceptibility and the *S.* enterica serovar. In humans, serovars Typhi and Paratyphi cause enteric fever that manifests one to 2 weeks following bacterial inoculation, while non-typhoidal *Salmonella* serovars such as *S.* Typhimurium and *S.* Enteritidis cause a localized gastroenteritis which results in diarrhoea, nausea, vomiting, intestinal cramping and fever within 12 to 72 hours after infection (Coburn *et al.*, 2006).

Salmonella infection starts with the ingestion of bacteria, usually through contaminated food. Following ingestion, *Salmonella* is able to resist the acidic environment in the stomach and the bactericidal effect of compounds such as bile salts, especially in the initial part of the small intestine. In this location, *Salmonella* reaches its main target, the gut associated lymphoid tissue of the ileum, in particular the M cells foci of the Peyer´s patches. Virulence genes of the TTSS-1 (type three secretion system) encoded in the *Salmonella* pathogenicity island I allow bacteria internalization in these M cells and also in enterocytes and goblet cells via a process called macropinocytosis (a form of endocytosis of large particles such as bacteria) (Francis *et al.*, 1993; Ginocchio *et al.*, 1994). Acting as a molecular syringe, the TTSS-1 injects proteins termed effectors into host cells (Fu and Galán, 1998). Four TTSS-1 effectors, named SipA, SopB, SopD and SopE2 act in concert to trigger alterations in the actin cytoskeleton of host cells, thereby promoting intestinal inflammation (Zhang *et al.*, 2002; Raffatellu *et al.*, 2004). Bacterial translocation across the intestinal epithelium occurs through the uptake by M cells as well as dendritic cells gaining access to the host circulation. Besides, numerous evidences indicate that *Salmonella* is able to disrupt tight junctions between

epithelial cells allowing paracellular transit into the gastrointestinal tissue (Boyle *et al.*, 2006).(Fig.1 and Fig.2)

An important factor in *Salmonella* pathogenesis is the ability to survive inside cells. Upon bacterial internalization or uptake, *Salmonella* resides in a unique membrane-bound compartment, different from the phagosome or lysosome, named SCV (salmonella containing vacuole). In this stage, the second type three secretion system (TTSS-2) codified in the SPI-2 inhibits the fusion with lysosomes and host oxidase killing (Raffatellu *et al.*, 2004; Thiennimitr *et al.*, 2012).

Once the epithelial barrier is breached, the innate immune cells stimulate the pattern recognition receptors (PRRs). Bacterial LPS, curli fimbriae, lipopeptide and flagellin (the monomeric subunit of the bacterial flagellar apparatus) are important PAMPs (pathogen-associated molecular patterns) recognized by host cells. The activation of Toll-like receptor 4 (TLR4) in response to *Salmonella* LPS causes a high inflammatory response in macrophages. Flagellin is recognized by TLR5 in epithelial cells and is a potent inducer of IL-8 (Gewirtz *et al.*, 2000; Vazquez-Torres *et al.*, 2004; Zeng, 2005). TLR2 in concert with TLR1 or TLR6 recognizes triacyl or diacyl bacterial lipopeptide. Curli fimbriae is the main PAMP detected by TLR1/TLR2 on intact bacterial cells (Tükel *et al.*, 2010).

In addition to the recognition of PAMPS from extracellular bacteria, the presence of intracellular *Salmonella* also leads to changes in the expression of host genes encoding for cytokines, cell surface receptors, or transcriptional activators. Upon phagocytosis of *Salmonella,* macrophages and dendritic cells activate a proinflammatory response, characterized by IL-12, IFN-γ and TNF-α production in mice. IFN-γ production is necessary to control bacterial replication, and is triggered by IL-12 produced by antigen-presenting cells (APCs). On the other hand, TNF-α enhances microbicidal activity synergistically with IFN-γ and triggers the production of nitric oxide.

The immune response against intracellular *Salmonella* has been shown to be very relevant for resistance against infection (Thiennimitr *et al.*, 2012) (Coburn *et al.*, 2006) (Eckmann and Kagnoff, 2001).

In the case of enterocolitis, upon colonization of the intestine, bacteria localize to the apical epithelium, induce invasion associated virulence factors and elicit significant inflammatory changes including polymorphonuclear cells (PMNs) and monocyte infiltration, inflammation of the lamina propia, epithelial necrosis, edema and fluid secretion (Fig. 1). Enterocolitis is more severe in the caudal ileum, the cecum and the proximal colon (Finlay *et al.*, 1989).

In the case of typhoid fever, bacteria disseminate via the mononuclear phagocyte system and form granulomatous foci in the liver and spleen known as typhoid nodules predominantly within macrophages, dendritic cells and PMNs (Coburn *et al.*, 2006; Wallis and Galyov, 2000; Raffatellu *et al.*, 2008). Spread to the gall bladder or urinary bladder can eventually also occur leading to chronic carriage, which is important for human-to-human spread of the disease (Gonzalez-Escobedo and Gunn, 2013) (Fig. 2). In the murine infection model, which is commonly accepted to study *Salmonella* pathogenesis due to its low cost and ease of use, *S.* Enteritidis and *S.* Typhimurium develop a systemic disease that resembles the enteric fever caused by *S.* Typhi in humans (Tsolis *et al.*, 2011).

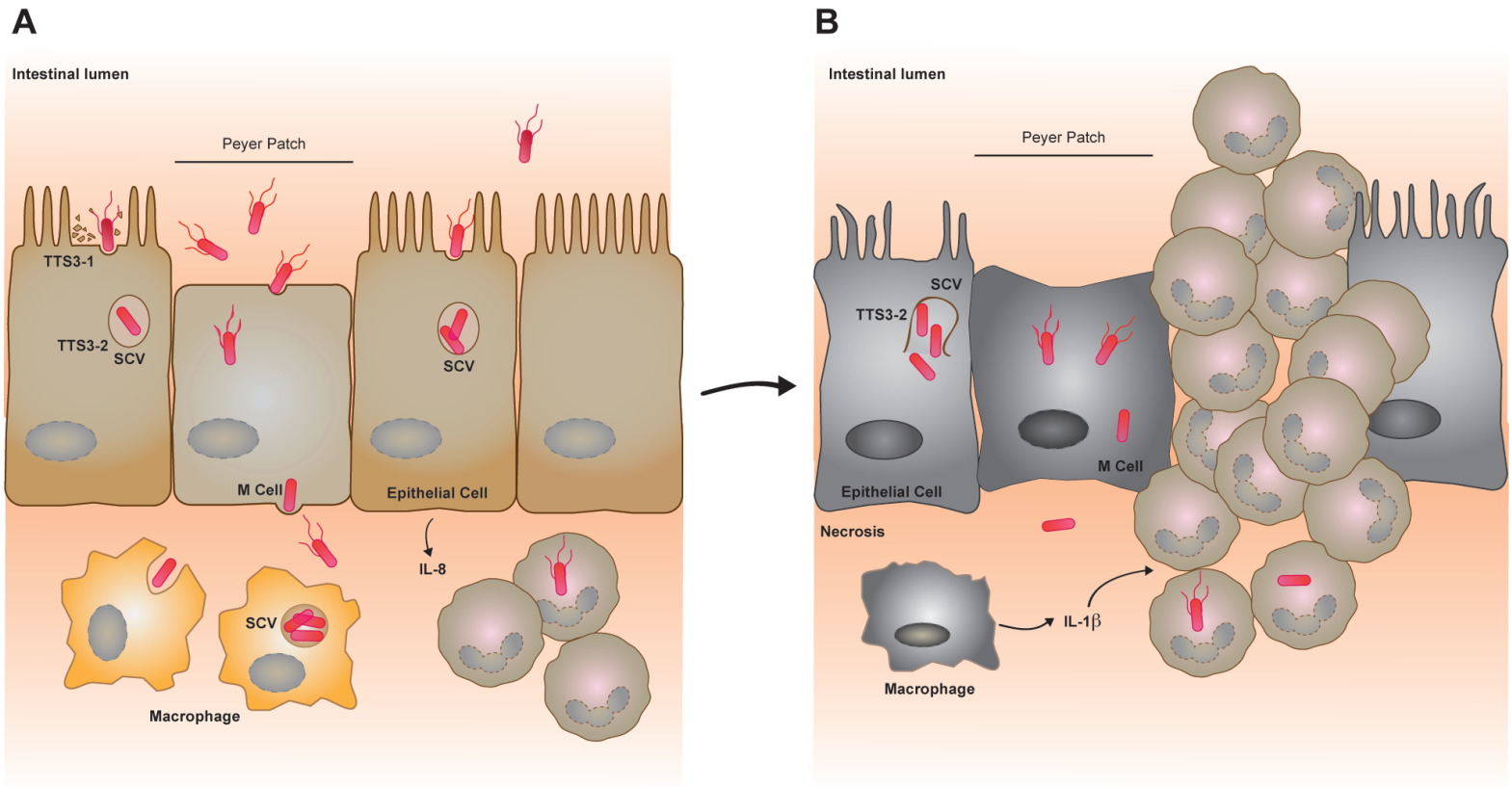


Fig. 1. Schematic representation of *Salmonella*-caused enterocolitis. A) *Salmonella* adheres to the intestinal epithelium and initiates the secretion of effector proteins from the TTSS-1 into the cell cytoplasm provoking its internalization in intestinal cells. Once inside the cell, *Salmonella* is housed in the SCV (*Salmonella* containing vacuole), where TTSS-2 effector proteins allow it to survive, replicate and finally migrate to the enterocyte basal wall. In response to bacterial infection, enterocytes produce IL-8 causing neutrophils recruitment to the lamina propia. These neutrophils alongside resident macrophages, phagocytose bacteria located in the lamina propia. B) Within macrophages, bacteria induce cellular death, fact that leads to IL1-β secretion and therefore to an increased inflammatory process. As inflammation progresses, PMNs migrate to the intestinal lumen across the epithelium while proteases and other inflammatory mediators favor the secretion of fluids, setting a diarrheal process.

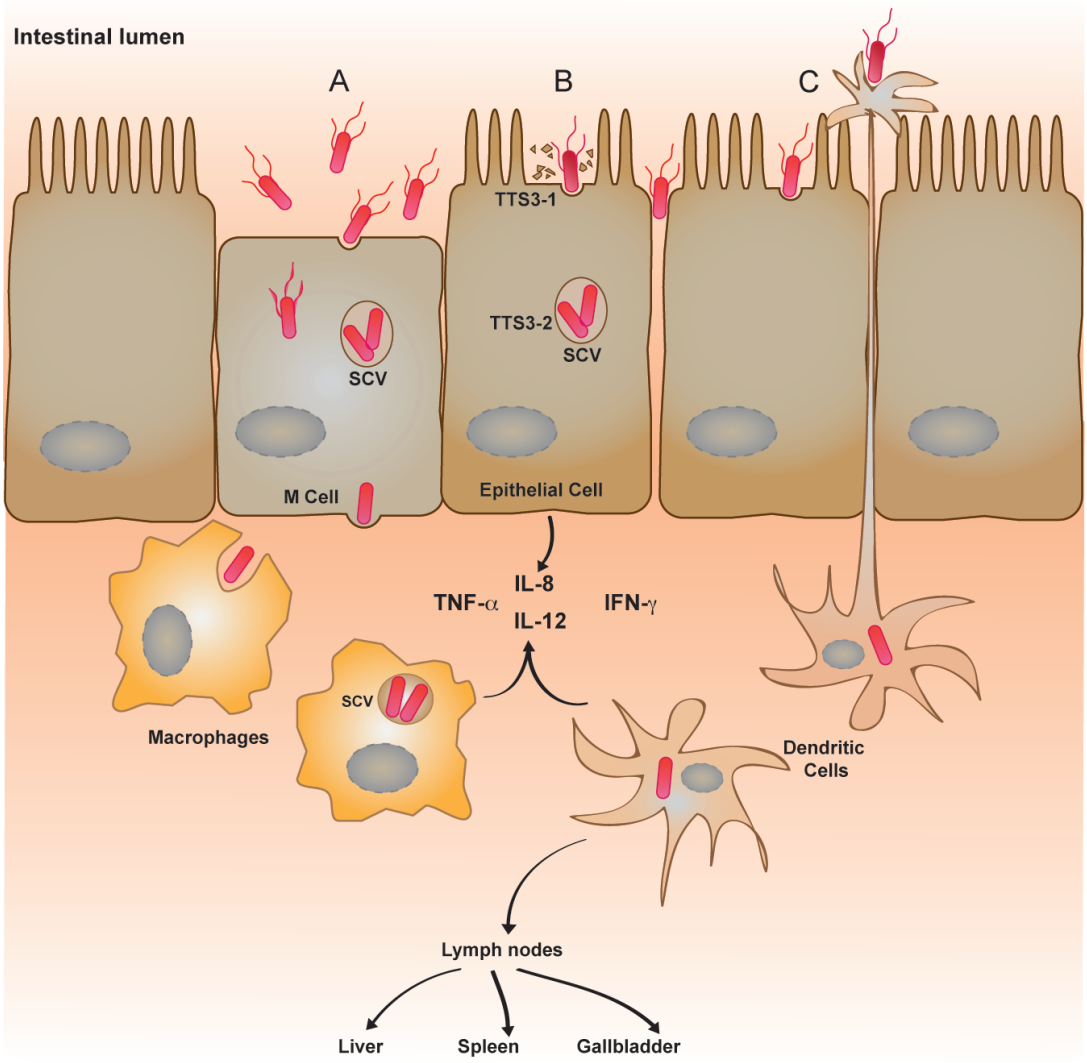


Fig. 2. Schematic representation of systemic dissemination of *Salmonella* in the murine model. The two mechanisms normally used by *Salmonella* to traverse the intestinal epithelium include the invasion of M cells (A) and pathogen capture by dendritic cells (C). However, *Salmonella* is also capable to penetrate enterocytes (B). Within dendritic cells, the enteropathogen reaches mesenteric nodules, where bacteria are transferred to macrophages and migrate to the spleen and liver via the lymph nodes. In target organs, *Salmonella* reside and replicate within macrophages.

Salmonella vaccines

Considering the worldwide incidence of *Salmonella* described above, a major objective for the prevention of *Salmonella* infections is to develop efficient vaccines (Mastroeni *et al.*, 2000). Currently available vaccines against salmonellosis can be divided into three major classes.

Whole-cell killed vaccines

These vaccines contain *Salmonella* cells inactivated with different procedures such as heat, phenol, acetone or formaldehyde treatment. These vaccines ensure a lower epidemiological risk than live vaccines and have the potential for inducing high humoral antibody titres. However, in contrast to live vaccines, an important weakness of inactivated vaccines is the lack of cell-mediated immunity induction, which is required for protection against the intracellular lifestyle of *Salmonella* (Harrison *et al.*, 1997).

Subunit vaccines

Instead of the entire microbe, subunit vaccines include only selected antigens that best stimulate the immune system. In some cases, these vaccines include specific parts of the antigen that antibodies or T cells recognize and bind to. Because subunit vaccines only contain the essential antigens and not all other molecules that make up the microbe, chances of adverse reactions to the vaccine are low.

An example of a subunit vaccine is the vaccine for *S.* Typhi registered as Typhim Vi. This vaccine is based on the immunogenicity that induces the capsule (Vi) that surrounds the bacteria and is unique to this serovar (Guzman *et al.*, 2006). Other subunit vaccines such as the ones based on detoxified LPS, cell extracts, porins, O-polysaccharide and O-conjugates

have been tested in experimental models and have shown lower efficiency (Mastroeni, 2003).

Live attenuated vaccines

Live attenuated vaccines contain a version of the living microbe that has been weakened in the lab so it cannot cause disease. Because an attenuated vaccine is the closest thing to a natural infection, the administration route usually coincides with the natural infection route of *Salmonella*. These vaccines elicit strong cellular and antibody responses and often confer lifelong immunity with only one or two doses. Despite their advantages, attenuated vaccines also have some downsides. The possibility, though remote, exists that an attenuated microbe in the vaccine could revert to a virulent form and cause disease. Also, live vaccines cannot be administered to immunocompromised people who have damaged or weakened immune systems. There are several vaccines based on live attenuated *Salmonella* strains approved for vaccination of humans or farm animals. These vaccines have been generally obtained by serial passages in the laboratory or through random mutagenesis, so the exact biological nature of the attenuation remains unknown. Nowadays, the availability of complete genome sequences together with new tools for genome modification and for identification of virulence genes expressed *in vivo* allows the design and generation of *Salmonella* mutants with very precise modifications to cause attenuation that can serve as potential vaccine candidates. Common strategies for attenuation of *Salmonella* are based on auxotrophies raised by mutations of genes involved in the synthesis of purines (*purA*, *purE* mutants) (Williams, 1956) or aromatic compounds synthesis (*aro* genes) (Callaghan *et al.*, 1990). Also, genes that are directly involved in the infection process, for example virulence genes encoded in pathogenity islands and the virulence plasmid, have been proposed for

attenuation purposes (Bohez *et al.*, 2008; Matulova *et al.*, 2012). Another common strategy is the mutation of global regulators like PhoPQ, or RpoS. The first one is a two-component system which regulates the expression of at least 40 virulence genes (Miller *et al.*, 1989). Some of these are involved in intramacrophage survival (Fields *et al.*, 1986) and resistance to host antimicrobial peptides (Groisman *et al.*, 1992; Beuzon *et al.*, 2001). RpoS is a global transcriptional regulator activated in response to different environmental conditions and represents the master regulator of the general stress response, playing a central role in the survival of bacteria under starvation or stress conditions, such as oxidative stress, DNA damage, and low pH, which *Salmonella* likely encounters during intracellular growth in host macrophages (Fang *et al.*, 1992) (Dong and Schellhorn, 2010). This global regulator plays a critical role in *Salmonella* virulence. For example, curli production (*csgBAC*), which is important for host colonization, is positively regulated by RpoS (Olsen *et al.*, 1993). RpoS also activates the expression of the plasmid-borne *spvR* and *spvABCD* genes, which are required for intracellular growth and systemic infection in mice and humans (Fang *et al.*, 1992).

A summary of the *Salmonella* vaccines available for chicken, pigs and humans is presented in Table 2.

Table 2. Commercial vaccines for chicken, pigs and human protection against *Salmonella*

Vaccine Name	Vaccine type	Animal in which the vaccine confers protection	Serotype of the vaccine strain	Attenuation mechanism	Serotype against which the vaccine confers protection	Company
NOBILIS® SG 9R CEVA® SG 9R	Live attenuated	Chicken	*S.* Gallinarium	Undetermined	*S.* Gallinarium	MSD Animal Health CEVA
NOBILIS® SALMOVAC440	Live attenuated	Chicken	*S.* Enteritidis	Undetermined	*S.* Enteritidis *S.* Typhimurium	IDT Biologika
NOBILIS® SALENVAC	Inactivated	Chicken	*S.* Enteritidis *S.* Typhimurium	Undetermined	*S.* Enteritidis *S.* Typhimurium	MSD Animal Health
NOBILIS® SALENVAC T	Inactivated	Chicken	*S.* Enteritidis *S.* Typhimurium	Undetermined. Addition of new inmunogens from *S.* enterica PT14 and *S.* Typhimurium DT104 strains to Salenvac vaccine.	*S.* Enteritidis *S.* Typhimurium *S.* Heilderberg *S.* Agona	MSD Animal Health
SALMUNE®	Live attenuated	Chicken	*S.* Typhimurium	Undetermined. Chromosome chemically mutated	*S.* Enteritidis *S.* Typhimurium *S.* Hadar *S.* Kentucky *S.* Heilderberg	CEVA
Layermune ®SE	Inactivated	Chicken	*S.* Enteritidis	Undetermined	*S.* Enteritidis	CEVA
AviPro® Salmonella VacE	Live attenuated	Chicken	*S.* Enteritidis	Mutant in essential enzymes and metabolic compartments due to a spontaneous mutation. Metabolic Drift Mutation (MDM).	*S.* Enteritidis	Lohmann

Vaccine Name	Vaccine type	Animal in which the vaccine confers protection	Serotype of the vaccine strain	Attenuation mechanism	Serotype against which the vaccine confers protection	Company
AviPro® Salmonella VacT	Live attenuated	Chicken	*S.* Typhimurium	Mutant in essential enzymes and metabolic compartments due to a spontaneous mutation. Metabolic Drift Mutation (MDM).	*S.* Typhimurium	Lohmann
AviPro® Salmonella DUO	Live attenuated	Chicken	*S.* Enteritidis *S.* Typhimurium	Mixture of AviPro® VacE Salmonella and AviPro®Salmonella VacT vaccines.	*S.* Enteritidis *S.* Typhimurium	Lohmann
Enterisol SC-54	Live attenuated	Pigs	*S.* Typhimurium	Undetermined	*S.* Typhimurium	BIVI
Enterisol Salmonella T/C	Live attenuated	Pigs	*S.* Cholerasuis *S.* Typhimurium	Undetermined	*S.* Cholerasuis *S.* Typhimurium	BIVI
SalmoPorc SCS	Live attenuated	Pigs	*S.* Cholerasuis	Undetermined	*S.* Cholerasuis	IDT Biologika
SalmoPorc STM	Live attenuated	Pigs	*S.* Typhimurium	Undetermined	*S.* Typhimurium	IDT Biologika
Vivotif	Live attenuated	Human	*S.* Typhi	Mutant in genes responsible for the production of Vi antigen and other undetermined genes.	*S.* Typhi	PaxVax
Typhim Vi	Subunit	Human	*S.* Typhi	Capsular polysaccharide Vi	*S.* Typhi	Sanofi Pasteur MSD
Peda-typhTM	Conjugated	Human	*S.* Typhi	Capsular polysaccharide Vi conjugated with Tetanus toxoid protein	S. Typhi and Clostridium tetani	BioMed

Bacterial Biofilms

Definition and overview

Biofilms are nowadays regarded as the major reservoirs of bacteria and other microbes in the environment. In the seventeenth century, Van Leeuwenhoek examined the “animalcules” in the plaque of his own teeth, but it was not until 1978 that the general theory of biofilm predominance was promulgated. In this year, J. William Costerton defined a biofilm as “bacteria covered by a glycocalyx of fibers that adhere to surfaces and to other cells” (Costerton *et al.*, 1978). Thirty-eight years later, the biofilm concept has evolved and is currently defined as aggregates of microorganisms that are attached to each other and/or to a surface, in which cells are embedded in a self-produced matrix of extracellular polymeric substance (EPS) (Flemming *et al.,* 2016) .

Biofilms are one of the most widely distributed and successful models of life on Earth. They comprise complex systems with high cell densities, ranging from 10^8 to 10^{11} cells g $^{-1}$ wet weigh, and typically include different bacterial species (Morgan-Sagastume *et al.*, 2008). Microbial communities can contain hundreds of strains and species, and we are only beginning to understand how and why different genotypes arrange themselves in space. Biofilm-dwelling cells influence each other’s evolutionary fitness through social phenotypes. Many of these phenotypes are simple forms of cooperation that benefit neighboring cells through the secretion of molecules such as digestive enzymes, nutrient chelators, wetting agents, structural polymers and signaling molecules (Zelezniak *et al.*, 2015). Owing to this cooperative and collective behaviour, bacteria within the biofilm have substantial advantages compared with solitary cells, including an increased resilience against external threats and a higher efficiency in digesting complex nutrients.

However, social interactions can also be competitive and cells within a microbial community should not be assumed to work harmoniously together. Competition for limited space and resources is very common, and many social phenotypes are dedicated to kill other strains and species. For instance, antibiotic secretion or injection of toxins into adjacent cells are mechanisms used by some species to avoid the growth of neighbor cells (Nadell *et al.*, 2016).

Harmful and beneficial biofilms

Biofilm formation has substantial implications in fields ranging from industrial processes to health-related subjects like medicine or dentistry. In some cases, this bacterial way of life results in beneficial effects. For example, bacterial biofilms are used to degrade toxic compounds such as petrol derivatives, heavy metals and soil bioremediation (Bums and Stach, 2002; Vu *et al.*, 2009; Meliani, 2014) or to improve waste water clean up (Smith *et al.*, 2015). In other cases, biofilms act as efficient biocontrol agents through the formation of microbial aggregates in the plant rhizosphere (Rudrappa *et al.*, 2008) and the subsequent increase in the productivity of important leguminous plants (Espinosa-Urgel, 2004). In healthcare, an attractive proposal concerning beneficial biofilms is to administer probiotics capable of forming biofilms, thus preventing colonization of pathogenic bacteria on the tooth surface, in the vagina and digestive tract (Vuotto *et al.*, 2014).

However, more often, biofilm formation is associated with great detrimental effects on industrial processes like oil drilling, paper production and food processing (Shi and Zhu, 2009; Van Houdt and Michiels, 2010). Also, biofilm formation on the surface of catheters, medical devices or host tissues correlates with deep-rooted chronic infections and resistance to both

phagocytosis and antimicrobial agents (Donlan, 2011; Donlan and Costerton, 2002).

Biofilm formation

The transition from a planktonic to a biofilm lifestyle occurs in response to environmental changes and involves multiple regulatory networks, necessary to translate environmental cues to the cellular machinery. In a first step, motile or non-motile bacteria attach irreversibly to a surface. Upon attachment, bacteria divide and daughter cells extend around the surface creating a colony. At this stage, bacteria initiate the synthesis of the extracellular matrix that surrounds the cells during biofilm growth. Finally, some bacteria are released from the biofilm community and the cycle starts again (Fig. 3).

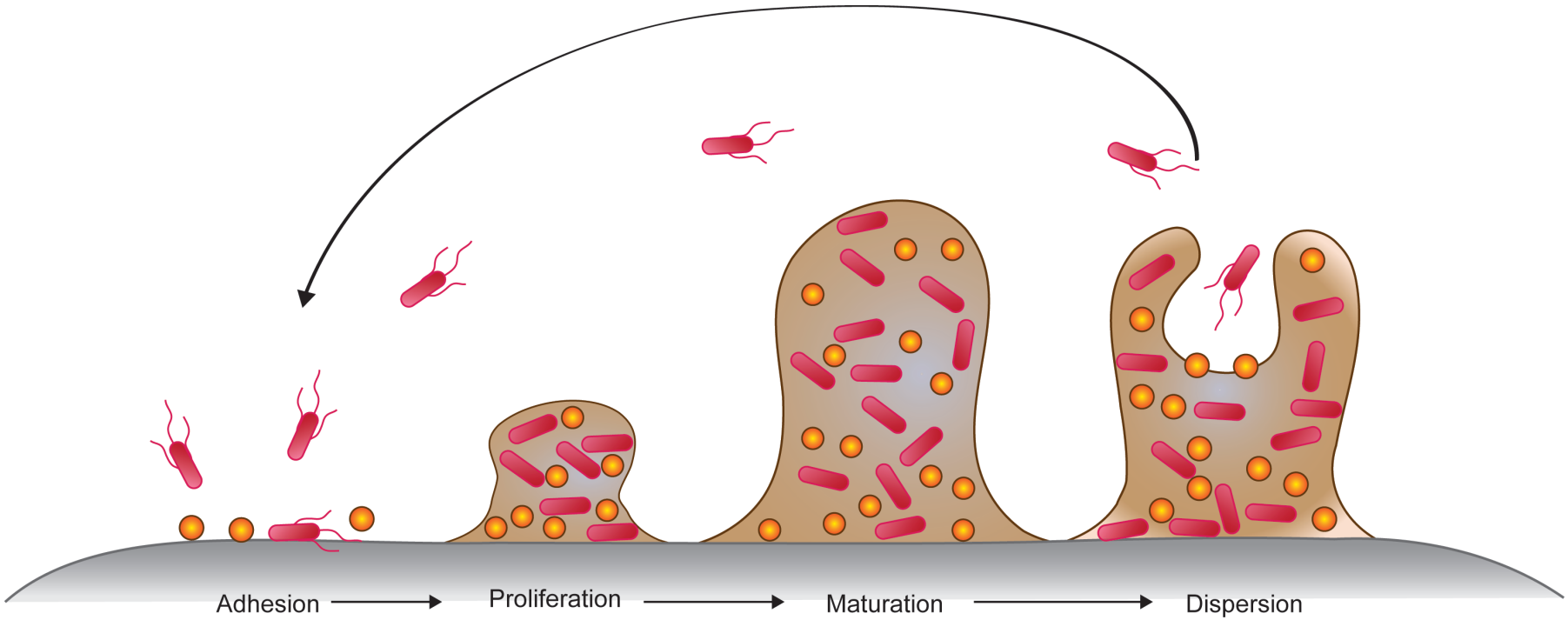


Fig. 3. Stages in the biofilm formation process. Biofilms develop via initial attachment, which depends on transport of the bacteria to a surface that is a passive process in the case of nonmotile bacteria such as staphylococci (yellow), and active in the case of motile bacteria such as *Salmonella* (pink). Initial attachment to biotic and abiotic surfaces depends on bacterial surface structures, like pili or adhesines. Subsequent steps do not differ in principle for motile or nonmotile bacteria. They involve proliferation, embedding in an extracellular matrix, and maturation. Finally, cells can eventually detach from the biofilm and re-enter a planktonic mode. These planktonic cells can then repeat the cycle, infecting new surface.

Bacterial living inside biofilms

The biofilm lifestyle is clearly distinct from that of free-living bacterial cells. Biofilm communities confer unique properties on bacteria since “group behavior” facilitates survival in adverse environmental conditions. Biofilms confer resistance to many external unfavorable environmental changes and to the host immune system, increase tolerance to antimicrobials and also promote the establishment of physical and social interactions that favor gene exchange (Flemming *et al.*, 2016).

A major element responsible for the increased protection to unfavorable conditions within the biofilm is the extracellular matrix that acts as a potent shield against extracellular aggressions whilst it allows water, nutrients and oxygen circulation through microscopic channels (Flemming *et al.*, 2016).

The biofilm extracellular matrix

The composition of the biofilm extracellular matrix is complex and variable, even within the same bacterial species when environmental conditions are altered. However, one common principle is that the matrix scaffold is built of exopolysaccharides and/or proteins, which eventually can be interwoven with extracellular genomic DNA. The reasons underlying the election of a polysaccharide or protein-based biofilm matrix are not well understood. In the next section we will focus on the exopolysacharidic biofilm matrix.

Despite the diversity of exopolysaccharides that bacteria are able to produce, there are two exopolysaccharides that different bacterial species have chosen for building the biofilm matrix; these are cellulose and poly-N-acetylglucosamine (PNAG/PGA) (Table 3). Interestingly, some bacteria, including *E. coli, Burkholderia cepanocepacia or Klebsiella pneumoniae,* have the capacity to produce both of them. It is assumed that each

exopolysaccharide is produced in a different environmental condition likely because it bestows different properties to the surrounded bacteria. However, this hypothesis has never been tested. This situation does not occur only with cellulose and PNAG/PGA exopolysaccharides. *Pseudomonas aeruginosa* can produce at least three types of polysaccharides, namely alginate, Psl and Pel and again, evidences suggest that the synthesis of these exopolysaccharides is tightly regulated and that they are produced under specific conditions. The finding that bacteria make use of different exopolysaccharides, depending on the environmental conditions, to build the biofilm matrix together with the fact that many genetically unrelated bacteria coincide in the same exopolysaccharide for this purpose strongly suggest that biofilm matrix exopolysaccharides have evolved in bacteria through strong selecting factors that are still not well understood.

A detailed description of the two most abundant exopolysaccharides used by pathogenic bacteria to build the biofilm matrix is summarized below.

Cellulose

Cellulose is one of the most abundant organic polymers in nature and the capacity to synthesize cellulose is widespread in bacteria (Table 3). Cellulose comprises only one type of sugar (glucose), which are linked together through β-1,4 linkages linearly. Bunches of β-1,4 glucan chains are assembled into microfibrils in the same direction, and then crystallized into cellulose fibers (Koyama *et al.*, 1997). In *E. coli* and *Salmonella*, the synthesis of this polysaccharide is regulated by two divergently operons *bcsABZC* and *bcsEFG* (Solano *et al.*, 2002). Due to the high interest on bacterial cellulose for biotechnological purposes, an enormous amount of studies has been devoted to investigate the properties of bacterial cellulose itself. However, only few studies have been focused on how cellulose is actually produced in bacteria. As a consequence, the exact role of each of

the proteins in the cellulose synthase machinery still remains poorly characterized. BcsA is an integral inner membrane protein with multiple transmembrane domains (Kumar and Turner, 2015) and a cytoplasmic family 2-glycosil-transferase domain. This protein catalyzes cellulose polymerization and facilitates translocation of the newly formed polymer across the inner membrane. In addition, it contains a PilZ domain in the C-terminal domain implicated in cyclic di-GMP (c-di-GMP) binding. Binding of the dinucleotide c-di-GMP to BcsA allosterically activates cellulose synthesis. BcsB is a periplasmic protein attached to BcsA through a single terminal TM helix and contains two carbohydrate binding domains (CBD1 and CBD2) that chaperone the synthesized glucan chain through the periplasm (Morgan *et al.*, 2013; Morgan *et al.*, 2016). The BcsA/BcsB complex has been shown to be sufficient for cellulose biosynthesis *in vitro* (Omadjelaa *et al.*, 2013). On the other hand, BcsC is a periplasmic protein that consists of an N-terminal α-helical part formed by several tetratricopeptide repeat (TPR) domains and a C-terminal part that is structurally similar to the β-barrels of outer membrane proteins. The TPR-containing N-terminal part of BcsC is believed to interact with peptidoglycan and other BCS components, while its C-terminal β-barrel domain is likely located in the outer membrane, forming a channel that guides the nascent glucan out of the cell (Whitney and Howell, 2013). Finally, BcsZ is a periplasmic protein, with endo β-1,4 glucanase activity and has been suggested to be required for degradation of cellulose chains in the periplasm and/or cleavage of nascent cellulose chains to allow microfibrill formation outside the cytoplasm (Römling and Galperin, 2015). The function of BcsE, BcsF, and BcsG proteins encoded in the second operon remains unknown, though a recent study has revealed that BcsE also binds c-di-GMP through a novel domain denominated GIL (GGDEF I site like), being

this binding essential for maximum cellulose production in *Salmonella* (Xin Fang *et al.*, 2014).

The membrane-integrated bacterial cellulose synthase contains the inner membrane components BcsA and BcsB as well as the outer membrane protein BcsC. Bacterial cellulose synthase polymerizes glucose via β-1,4 glycosidic linkages in a multi-step process which requires the presence of a divalent cation, usually magnesium, and c-di-GMP. First, upon stimulation by c-di-GMP, the synthase binds its substrate UDP-Glc (donor) at an intracellular glycosyltransferase domain. Second, the donor glucose is transferred to the 4´hydroxyl group at the non-reducing end of the growing polysaccharide chain (acceptor), thereby extending the polymer and forming UDP as a second reaction product. Third, following glycosilic transfer, the elongated polymer has to be translocated by one glucose unit into a transmembrane (TM) channel so that the newly added glucose unit occupies the acceptor site and UDP must be replaced with UDP-Glc for another round of catalysis (Fig. 4) (Morgan *et al.*, 2014).

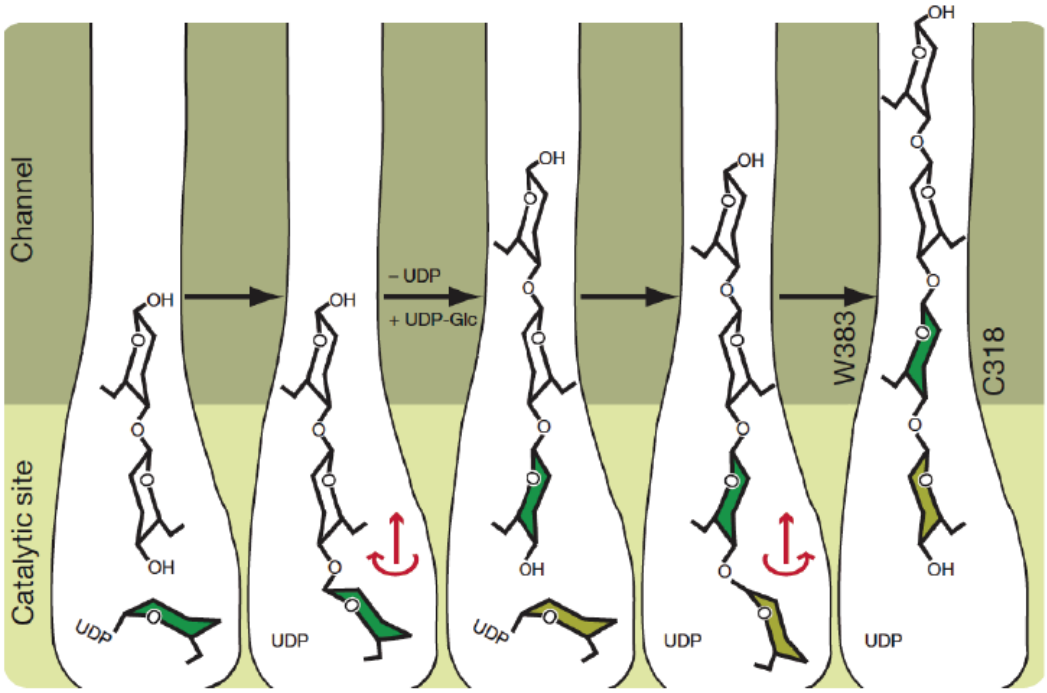


Fig. 4. Proposed model for cellulose synthesis and translocation (Morgan *et al.,* 2013) After glycosyl transfer, the newly added Glc could rotate around the acetal linkage into the plane of the polymer. The rotation direction would be determined by steric interactions and formation of the β-1,4 glucan characteristic intramolecular O3-H-O5 hydrogen bond. The glucan might translocate into the channel during this relaxation. This process would be repeated with a second UDP-Glc but the rotation direction after glycosyl transfer would be in the opposite direction owing to steric constraints. Alternatively, the glucan might not translocate into the channel until UDP is replaced by UDP-Glc. Trp 383 and Cys 318 mark the entrance to the transmembrane channel (only shown in the right panel).

The outstanding crystallographic work with *Rodobacter sphaeroides* BcsA-BcsB complex developed by (Morgan *et al.*, 2014) has confirmed a theoretical mechanistic model on the allosteric activation of cellulose synthesis by c-di-GMP (Benach *et al.*, 2007; Ko *et al.*, 2010; Fujiwara *et al.*, 2013; Omadjelaa *et al.*, 2013). BcsA contains eight transmembrane (TM) helices and a cytosolic glycosyltransferase (GT) domain between TM helices four and five. The GT domain contains seven β-barrel surrounded by seven α-helix and linked to the transmembrane region via three amphipathic interface (IF) helices (IF1-3). It is important to note that IF3 forms a loop with TM7 called the gating loop. Between the TM3-8 domains, BcsA also forms a polysaccharide channel across the membrane, thereby allowing the coupling of cellulose synthesis and translocation. Finally, BcsA contains a PilZ domain within its C-terminal intracellular extension, which comprises an RRxxxR motif in a flexible linker region followed by a β-barrel that contains a DxSxxG motif, both responsible of c-di-GMP binding. In the presence of c-di-GMP, one molecule of the dimer binds to the DxSxxG β-barrel motif and the other molecule to the RRxxxR TM8- β-barrel motif, being both arginine (Arg580) and (Arg584) implicated in c-di-GMP interaction. In the absence of c-di-GMP, Arg580 of the PilZ domain contacts Thr511 of the gating loop, which is located in front of the GT domain entry, blocking the catalytic site of the enzyme. Also a saline bridge between Arg580 and Glu371 stabilizes the autoinhibited state of the enzyme. In the presence of c-di-GMP, a conformational change of the molecule occurs. Arg580 rotates 180º to bind c-di-GMP and breaks its interaction with the gating loop and Glu371. Besides, the gating loop is stabilized by hydrophobic interactions with the amphipatic helix (IF) and forms the TM channel entry, leaving the UDP-Glc binding site available.

PIA-PNAG or PGA (poly N-acetyl-glucosamine)

This polysaccharide is a β-1,6-linked N-acetyl–D-glucosamine homopolymer that was firstly described in the gram-positive bacteria *Staphylococcus epidermis* (Heilmann *et al.*, 1996). Initially, it was named as PIA (polysaccharide intercellular adhesin) (Heilmann *et al.*, 1996). Later on and based on its composition the group of J. Pier named it as PNAG (McKenney *et al.*, 1998; Maira-Litran *et al.*, 2002). Because there is no agreement in the area with respect to this polysaccharide name, it will hereafter be denominated PIA/PNAG.

The genes involved in PIA/PNAG synthesis are encoded in the *icaADBC* operon (Cramton *et al.*, 1999). IcaAD form a complex responsible for the synthesis and export of the polysaccharide. IcaC functions by adding succinyl groups to the growing polymer using its O-succinyltransferase activity and, on the other hand, IcaB is an N-deacetylase that is specific to PIA-PNAG (Atkin *et al.*, 2014). An exopolysaccharide of identical composition to PIA-PNAG has been identified by the group of T. Romeo (Wang *et al.*, 2004) in *E. coli*. This exopolysaccharide was named PGA and according to the presence of orthologous genes in many different bacteria, it is also produced by many different gram negative bacteria (Table 3). In *E. coli* the synthesis of the PGA polysaccharide is driven by the proteins encoded in the *pgaABCD* operon (Wang *et al.*, 2004). While PgaC and PgaD are implicated in its synthesis, PgaA and PgaB are responsible for poly-GlcNA export (Itoh *et al.*, 2008). PgaC is the equivalent to IcaA, with a cytoplasmatic GT-2 domain that is 43% identical at the aminoacid level. PgaC is located in the inner membrane and polymerizes poly-GlcNAc from activated UDP-GlcNAc precursor being the catalytic domain exposed to the cytoplasm. PgaD is a small protein that forms a complex with PgaC to regulate polysaccharide synthesis. PgaA is an outer membrane porin that serves to translocate growing poly-GlcNAc chains to the cell surface (Itoh *et*

al., 2008). PgaB is a putative outer membrane lipoprotein that deacetylates about 3% of the GlcNAc residues during poly-GlcNAc export and shares the same function with IcaB (Wang *et al.*, 2004; Itoh *et al.*, 2008; Atkin *et al.*, 2014).

In the case of PGA synthesis, protein complex structures and regulation mechanisms are not as well defined as in the case of the cellulose polysaccharide. Similarly to the regulation of cellulose synthesis in gram negative bacteria, PGA synthesis is allosterically regulated by c-di-GMP in *E. coli* (Steiner *et al.*, 2013) (Fig. 5). The work of (Steiner *et al.*, 2013) has allowed a better understanding of the regulation of PGA synthesis by this dinucleotide. As stated before, in *E. coli*, poly-GlcNa is synthetized and secreted by the envelope-spanning PGA machinery, which is encoded in the *pgaABCD* operon (Wang *et al.*, 2004). PgaC is a processive β-glycosyltransferase (GT) of the GT-2 family that is located in the inner membrane and polymerizes poly-GlcNAc from activated UDP-GlcNAc precursor (Wang *et al.*, 2004; Itoh *et al.*, 2008). The catalytic domain of the GT-2 family is exposed to the cytoplasm with sugar transfer through the cytoplasmic membrane being independent of an undecaprenyl phosphate lipid carrier. In the presence of c-di-GMP, PgaC forms a stable complex with PgaD and binds c-di-GMP to activate polysaccharide synthesis. When the level of c-di-GMP decreases, PgaD is not bound to c-di-GMP and the protein adopts a conformation that is more prone to degradation by proteases (Steiner *et al.*, 2013). Using error prone PCR mutagenesis the authors found and isolated specific mutations (N75D, K76E) in PgaD and (V277L) in PgaC that mimic a c-di-GMP bound state that activates the PgaCD GT complex. They also studied the involvement of PgaC conserved arginines in c-di-GMP binding and found that Arg222 is implicated in c-di-GMP binding and complex formation with PgaD. However, future crystallographic studies may help to resolve the exact structure of

these proteins and the residues implicated in poly-GlcNa synthesis and c-di-GMP regulation.

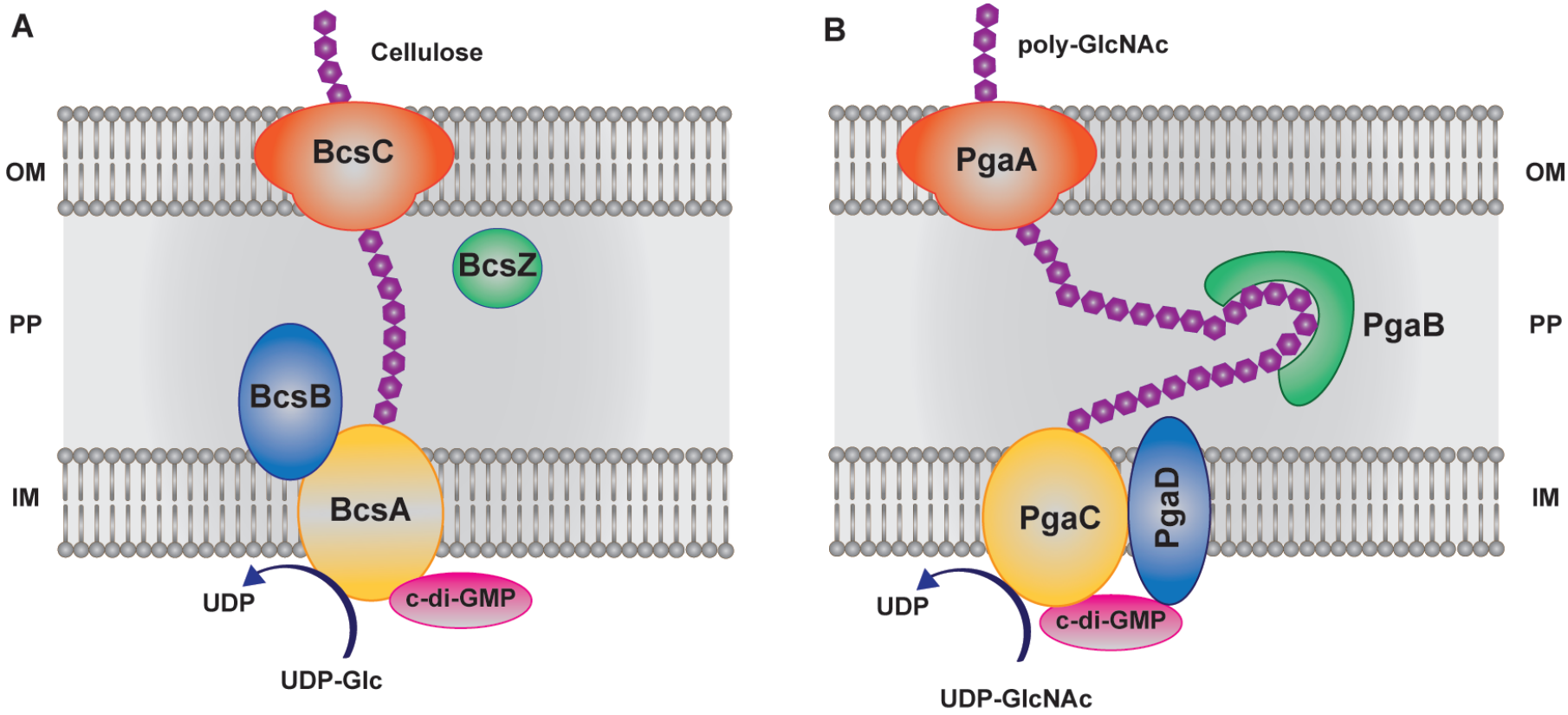


Fig. 5. Schematic representation of PGA and cellulose exopolysaccharides synthesis machineries. The components for A) cellulose synthesis machinery and B) PGA synthesis machinery are shown. Color-code is used according to a similar predicted function as follows: glycosiltransferase, yellow; porin, orange; exopolysaccharide-modifying enzyme, green; proteins that couple glycosiltransferase activity, blue. In each system, the polysaccharide indicated is polymerized and transported across the inner membrane by its respective synthase. For both polysaccharides the process also requires c-di-GMP, shown in pink. Once in the periplasm, polysaccharide-modifying enzymes act on each polysaccharide before they are exported across the outer membrane by the porin protein. IM, inner membrane; OM, outer membrane; PP, periplasmic space; UDP-Glc, UDP glucose; UDP-GlcNAc, UDP N-acetylglucosamine.

Table 3. Summary of the most common bacterial polysaccharides.

Bacteria	Polysaccharide	Type of pathogen	Reservoir	Gram stain	Reference
***Salmonella* spp**	Cellulose/ Colanic acid	Facultative intracellular human pathogen	Environment. Human and animal intestine	Negative	(Solano *et al.*, 2002) (Zogaj *et al.*, 2001)
***Citrobacter* spp**	Cellulose	Facultative intracellular human pathogen	Environment. Human and animal intestine	Negative	(Zogaj *et al.*, 2003)
***Burkholderia* spp**	Cellulose/PGA	Facultative intracellular human pathogen	Environment (soil)	Negative	(Yakandawala *et al.*, 2011) (Römling and Galperin, 2015)
***Escherichia* spp**	Cellulose/PGA/Colanic acid	Commensal and extracellular human pathogen	Environment. Human and animal intestine	Negative	(Zogaj *et al.*, 2001) (Wang *et al.*, 2004)
Acinetobacter baumanii	PGA	Facultative intracellular human pathogen	Environment. Human skin and respiratory tract	Negative	(Choi *et al.*, 2009)
Bordetella pertussis	PGA	Human pathogen	Humans	Negative	(Parise *et al.*, 2007)
Actinobacillus	PGA	Facultative intracellular human pathogen	Humans and animals	Negative	(Kaplan *et al.*, 2004)

Bacteria	Polysaccharide	Type of pathogen	Reservoir	Gram stain	Reference
***Yersinia* spp**	PGA	Facultative intracellular human pathogen	Fleas and rodents	Negative	(Bobrov *et al.*, 2008)
Vibrio cholerae	VPS	Extracellular human pathogen	Environment	Negative	(Yildiz and Schoolnik, 1999)
Klebsiella pneumoniae	Cellulose/PGA	Facultative intracellular human pathogen	Environment Nosocomial infections	Negative	(Römling, 2002) (Zogaj *et al.*, 2003) (Kuang-Ming Chen *et al.*, 2014)
Pseudomona aureginosa	Psl/Pel/Alginate	Extracellular human pathogen	Environment . Nosocomial infections.	Negative	(Franklin *et al.*, 2011)
Pseudomona putida	Pea/Peb/Alginate/ Cellulose	Plant symbiont	Environment	Negative	(Nilsson *et al.*, 2011)
Chromobacterium violaceum	Cellulose/PGA	Human pathogen	Environment	Negative	(Recouvreux *et al.*, 2008) (Becker *et al.*, 2009)
Enterobacter amnigenus GH-1	Cellulose	Human pathogen	Environment. Human intestine	Negative	(Hungund and Gupta, 2010)
Pseudomonas syringae	Cellulose	Plant pathogen	Environment	Negative	(Prada-Ramírez *et al.*, 2016)
Pseudomonas fluorescens	Cellulose	Plant symbiont	Environment	Negative	(Spiers *et al.*, 2003)

Bacteria	Polysaccharide	Type of pathogen	Reservoir	Gram stain	Reference
Rhizobium leguminosarum	Cellulose	Plant symbiont	Environment	Negative	(Laus *et al.*, 2005)
Erwinia chrysanthemi	Cellulose	Plant pathogen	Environment	Negative	(Römling, 2002)
Dickeya dadantii	Cellulose	Plant pathogen	Environment	Negative	(Jahn *et al.*, 2011)
Xhantomonas campestris	Xhantan	Plant pathogen	Environment	Negative	(Harding *et al.*, 1993)
Vibrio fischeri	Cellulose	Symbiont marine animals	Environment	Negative	(Bassis and Visick, 2010)
Agrobacterium tumefaciens	Cellulose/curdlan/acidic succinoglycan/ cyclic-β-1,2-D-glucan	Plant pathogen	Environment	Negative	(Matthysse *et al.*, 1981)
Komagataeibacter xylinus	Cellulose		Environment: soil	Negative	(Ross *et al.*, 1987)
Rodobacter sphaeroides	Cellulose		Aquatic environment	Negative	(Morgan *et al.*, 2013)
Sinorhizobium meliloti	(1→3)(1→4)- β-glucan	Plant symbiont	Environment	Negative	(Pérez-Mendoza *et al.*, 2015)
Mycobacterium tuberculosis	Cellulose	Facultative intracellular human pathogen	Human, respiratory tract	Positive	(Trivedi *et al.,* 2016)

Bacteria	Polysaccharide	Type of pathogen	Reservoir	Gram stain	Reference
Sarcina ventriculi	Cellulose	Human pathogen	Environment. Animal and human stomach	Positive	(Canale-Parola *et al.*, 1961)
***Stapylococcus* spp**	PGA	Extracellular human pathogen	Human nose, groin, axillae skin…	Positive	(Heilmann *et al.*, 1996)
Bacillus subtillis	PGA	Extracellular human pathogen	Soil, commensal gut.	Positive	(Roux *et al.*, 2015)
Lysteria monocytogenes	ManNAc-Gal EPS	Facultative intracellular human pathogen	Environment	Positive	(Köseoğlu *et al.*, 2015)

The Salmonella biofilm

Salmonella transition involving the environment-host-environment cycle depends enormously on the capacity to form biofilms. *Salmonella* is able to form biofilms on crops, soil, aquatic systems and on the surface of several materials like glass, polymer or steel (Woodward *et al.*, 2000; Hood and Zottola, 1997). In farms, *Salmonella* can form biofilms in waterways, feeders, animal faeces or even on egg surfaces (Vestby *et al.*, 2009; Brandl *et al.*, 2011; Brandl *et al.*, 2013; Patel *et al.*, 2013; Pande *et al.*, 2016). In food-processing environments, conventional cleaning and sanitation procedures usually fail to eradicate *Salmonella* biofilms (Vestby *et al.*, 2009; Shi and Zhu, 2009; Van Houdt and Michiels, 2010) that turn contaminated surfaces and equipment into a source of food contamination.

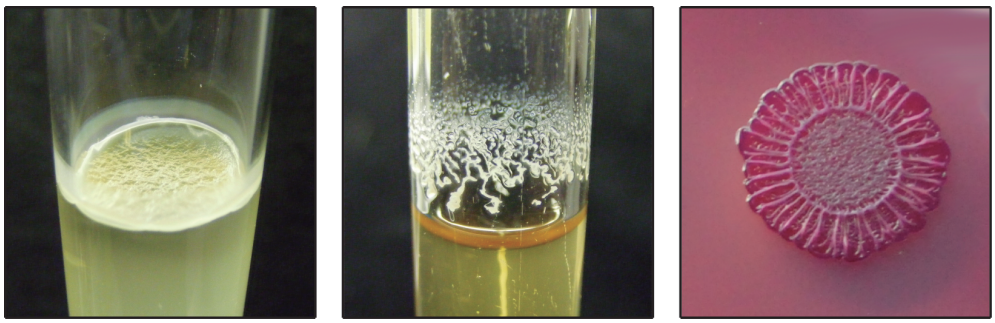
During human infection, *S.* Typhi can form biofilms in the gallbladder and produce chronic infections. In these patients (3-5% of infected people), *S.* Typhi is released through bacterial shedding in feces and urine. These infections can persist for long periods of time because the infected individuals, despite being highly contagious, are typically asymptomatic.

Taking into account the impact that *Salmonella* biofilms have on human health and industry, the study of the composition and regulation of the biofilm is of great interest.

Salmonella biofilm formation: components and regulation

The *Salmonella* biofilm exhibits different macroscopic phenotypes that vary according to the media and growth conditions used in the laboratory. When *Salmonella* is grown in rich media at room temperature without shaking, it produces a macroscopic pellicle on the interphase between the liquid and the air (Fig 6A). In contrast, when it is grown in a minimal defined media (ATM) at 37 °C under shaking conditions, a ring of bacteria adhered

to the surface of the tube is formed (Fig. 6B) (Solano *et al.*, 1998). On solid rich media, supplemented with the congo red dye, *Salmonella* colonies show a red dry and rough morphotype known as the rdar (red, dry and rough) phenotype (Romling, *et al.*, 1998) (Fig. 6C).



A) LB medium,
72 hours at room termperature

B) ATM medium
4 hours at 37ºC
under shaking conditions

C) Congo red agar
48 hours at 28ºC

Fig. 6. Different phenotypes displayed by a biofilm positive *S.* Enteritidis strain. A) Pellicle developed at the air-liquid interphase by a standing culture grown in LB after 72 hours of incubation at room temperature. B) Ring of bacterial cells adhered to the surface of a glass tube after 4 hours of incubation in ATM under vigorous shaking conditions. C) rdar morphotype on congo red agar plates after 48 hours of incubation at 28ºC.

The *Salmonella* biofilm extracellular matrix is essentially composed of three components: cellulose, curli fibers and the BapA protein. Cellulose has been described in a previous section of this introduction. Curli fibers are protein appendages formed by repetitions of a protein unit (CsgA) and play an important role in the initial adhesion to surfaces or host tissues. Curli fibers possess a cross β-sheet quaternary structure that is characteristic of amyloids in general and makes these proteins resistant to proteolytic digestion (Hufnagel *et al.*, 2013). They were initially described in *S.* Typhimurium as thin aggregative fimbriae and are encoded by the *csg* gene cluster that consist of *csgBAC* and *csgDEFG* genes (Romling *et al.*, 1998). Although all these genes are essential for fiber synthesis, the exact role of some of them in curli biogenesis remains unknown. On the other hand, BapA is the second largest protein of the *S.* Typhimurium genome. It can be included in a group of surface proteins that exhibit homology with Bap of *Staphylococcus aureus* (Biofilm-associated protein) and that promote adhesion to biotic and/or abiotic surfaces (Latasa *et al.*, 2006). One common aspect about Bap proteins is their large size mainly due to the presence of a core domain formed by a variable number of tandem repeats. Region B of BapA (aa 159–3003) contains 28 tandem imperfect repeats of 86–106 amino acids. Each repeat of BapA shows on average a 29% of identity with a C-repeat of Bap. According to protein domain prediction programs (ProDom structural genomics), region B of BapA also exhibits homology with a large repetitive RTX inner membrane family of exoproteins involved in adhesion and virulence processes (Latasa *et al.*, 2005).

The presence of these three components is necessary for a robust biofilm formation. Mutants deficient in any of these compounds are unable to form a biofilm though overexpression of curli fibers can compensate for BapA absence (Latasa *et al.*, 2005). *Salmonella* phenotypes under biofilm forming conditions of individual, double and triple mutants in these three

components implicated in biofilm formation are shown in Fig. 7.

The synthesis of all three elements, cellulose, curli and BapA is controlled by the LuxR type regulator CsgD. Thus, CsgD is the master transcriptional regulator of *Salmonella* biofilm assembly, acting as a positive regulator of the *csgBAC* operon to promote the transcription of the curli structural components CsgA and CsgB and also of the *bapA* gene (Romling *et al.*, 2000; Latasa *et al.*, 2005). CsgD also promotes cellulose production by transcriptionally activating *adrA*, a gene encoding a diguanilate cyclase that produces the amount of c-di-GMP necessary to allosterically activate BcsA (Romling *et al.*, 2000) (Fig. 8).

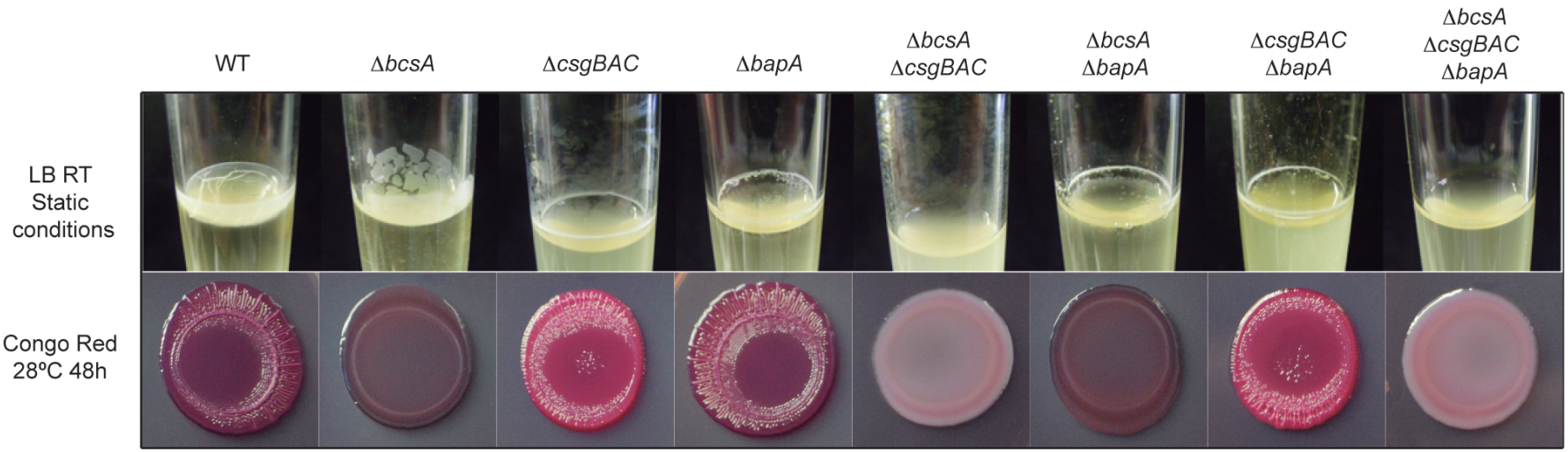


Fig. 7. Phenotypes displayed by single, double and triple mutants in genes encoding extracellular matrix components of the biofilm formed by *Salmonella*. The upper pannel shows the biofilm phenotype displayed after growth in LB media, for 72 hours at room temperature. A floating pellicle at the air–broth interface that totally blocks the surface of the culture is characteristic of a *Salmonella* biofilm. A pellicle of fragile appearance at the air–broth interface, which is easily disrupted by shaking, is indicative of cellulose absence. A lack of curli fimbriae and/or BapA leads to a complete non-biofilm forming phenotype. The bottom pannel shows the morphoype exhibited after incubation on congo red agar plates for 48 hours at 28ºC. A rdar (red, dry and rough) morphotype is characteristic of a *Salmonella* biofilm forming strain. Absence of cellulose gives rise to a bdar (brown, dry and rough) morphotype, whilst a curli fimbriae mutant shows a pdar (pink, dry and rough) morphotype. Absence of both cellulose and curli fimbriae leads to a saw (smooth and white) morphotype. BapA does not influence the *Salmonella* phenotype shown on congo red agar plates. *ΔbcsA*, cellulose mutant; *ΔcsgBAC*, curli mutant; *ΔbapA*, BapA mutant.

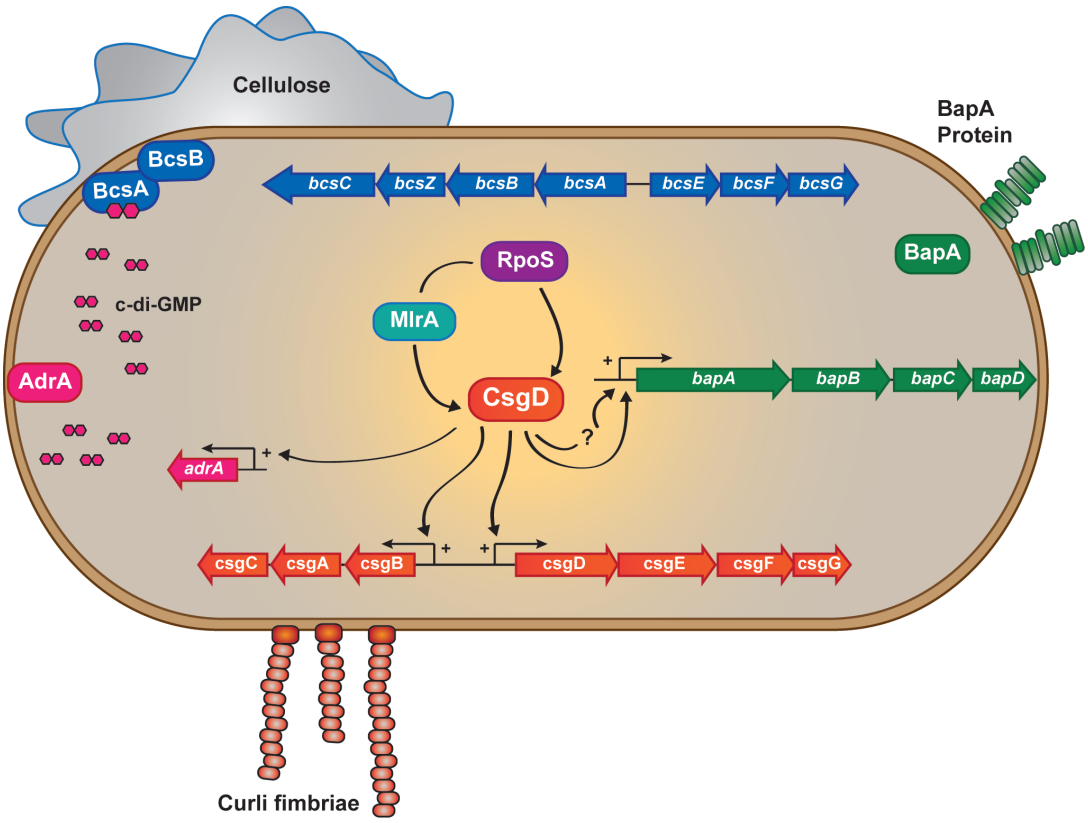


Fig. 8. Current model of the regulatory network controlling the expression of extracellular matrix components required for biofilm formation in *Salmonella*. CsgD is the *Salmonella* biofilm master regulator and its expression is controlled by the transcriptional global regulators RpoS and MlrA. CsgD positively regulates curli fimbriae synthesis, encoded in two divergently operons (represented in orange colour) and also activates cellulose production through the expression of AdrA, a member of the GGDEF protein family, which synthetizes c-di-GMP. C-di-GMP binding to the BcsA PilZ domain results in cellulose production. Genes encoding proteins needed for cellulose production are transcribed in two operons, *bcsABZC* and *bcsEFG* (shown in blue). The expression of the large secreted protein BapA is also regulated by CsgD, although the mode by which CsgD activates *bapABCD* operon (shown in green) transcription remains unknown.

The c-di-GMP signal transduction system

Discovery of c-di-GMP and system overview

A key factor that determines the evolutionary success of a bacterium is the capacity to sense environmental factors and to respond accordingly. Thus, all bacteria have evolved signal transduction mechanisms to establish "functional connectiveness" between environmental cues and cellular physiology. In the case of bacteria, two-component systems (TCSs) are the primary means of the sensorial machinery. A second signal transduction mechanism in bacteria involves the molecule 3',5'-cyclic diguanosine monophosphate (c-di-GMP) (Jenal and Malone, 2006; Cotter and Stibitz, 2007; Pesavento and Hengge, 2009; Schirmer and Jenal, 2009; Mills *et al.*, 2011; Romling *et al.*, 2013; Hengge *et al.*, 2015; Tamayo *et al.*, 2007; Povolotsky and Hengge, 2012; Hengge, 2013). Bacterial c-di-GMP was discovered in 1987 by the group of Moshe Benziman and coworkers, who reported c-di-GMP as an allosteric activator for cellulose synthase in *Gluconacetobacer xylinum* (Ross *et al.*, 1987). Ten years later, the same group identified the proteins implicated in c-di-GMP synthesis and degradation. They found that proteins that contain a GGDEF domain have diguanylate cyclase (DGC) activity and are able to synthesize c-di-GMP whereas proteins containing an EAL domain are c-di-GMP specific phosphodiesterases (PDEs) that degrade c-di-GMP (Tal *et al.*, 1998). Later on, a second domain involved in c-di-GMP degradation termed HD-GYP was described (Ryan *et al.*, 2006).

Genome sequencing has revealed the presence of genes encoding proteins containing domains associated with c-di-GMP signalling in most bacterial species. Pfam database (http://pfam.xfam.org) reveals the existence of more than 28.000 GGDEF proteins spread among 1.813 species and more than 17.000 EAL proteins in 1.591 bacterial species. Experimental evidence for c-di-GMP signalling pathways has been obtained

in the major phylogenetic branches, including Proteobacteria, Spirochetes, Cyanobacteria, Deinococcus-Thermus, Thermotogae, Actinobacteria and Firmicutes. Synthesis of c-di-GMP is not restricted to prokaryotic cells, and the amoeba *Dictyostelium discoideum* uses c-di-GMP in the cell differentiation process (Zhi-hui Chen and Schaap, 2012).

The number of proteins dedicated to c-di-GMP-mediated signalling is highly variable amongst bacteria. For example, the genome of *Escherichia coli* K-12 encodes 19 proteins with a GGDEF domain and 17 with an EAL domain, whereas *Bacillus subtilis* has four and three, respectively, and even the tiny genome of *Rickettsia prowazekii* encodes one of each. The bacteria with the largest number of these type of proteins is *Vibrio vulnificus*, that encodes 66 proteins with a GGDEF domain and 33 with an EAL domain (Galperin *et al.*, 2001; Galperin, 2004; Romling, 2005).

An interesting characteristic of GGDEF/EAL proteins is that most of them have a multimodular architecture. The GGDEF and EAL domains are often located C-terminally from multiple sensory and signal transduction domains. Common sensory domains found in proteins containing GGDEF and EAL domains include those termed GAF, PAS, HAMP, REC, BLUF, Lux-R like, MASE1, MASE 2 and HAMP (Galperin, 2005). These domains have been linked to the sensing of small molecules, redox potential, light, voltage, oxygen, nutrients, osmolarity, antibiotics, homoserine lactones, and a number of other signals. Based on this modular architecture, it has been proposed that GGDEF/EAL proteins are members of a new signal transduction system that uses c-di-GMP as a secondary messenger. Thus, different GGDEF proteins will sense different stimulus and in response to this signal will modify the levels of c-di-GMP which will subsequently affect different biological process such as the biosynthesis of adhesins and exopolysaccharides (Lee *et al.*, 2007; Wolfe and Visick, 2008), motility (Girgis *et al.*, 2007; Wolfe and Visick, 2008), long-term survival and

response to environmental stresses (Klebensberger *et al.*, 2007; Sabirova *et al.*, 2008), synthesis of secondary metabolites (Fineran *et al.*, 2007), regulated proteolysis and cell cycle progression (Duerig *et al.*, 2009) and virulence (Tamayo *et al.*, 2008; Lai *et al.*, 2009).

Synthesis and degradation of c-di-GMP by GGDEF, EAL and HD-GYP domain proteins

Diguanylate cyclases (DGCs)

DGC proteins form homodimers, where each GGDEF domain binds one molecule of GTP in an antiparallel position to form 3´-5´phophodiester linkages. These proteins contain two relevant enzymatic sites named A and I sites. The A site is the catalytic site of the protein (GGDEF) in which the two first residues are responsible for GTP binding, the third residue plays an important role in catalysis and the glutamic residue is involved in Mg^{2+} and Mn^{2+} ions binding. The I site (RxxD) is located five amino acids upstream of the GGDEF motif. When c-di-GMP binds to the I-site of a DGC, c-di-GMP synthesis is allosterically inhibited. This mechanism avoids GTP excessive consumption and elevated c-di-GMP accumulation (Ralf Paul *et al.*, 2007; Schirmer and Jenal, 2009).

Phosphodiesterases

Proteins with phosphodiesterase activity are characterized by containing the EAL or HD-GYP motifs. EAL proteins hydrolyze the c-di-GMP molecule to 5´-pGpG, that is later degraded to two GTP molecules by the oligoribonuclease Orn (Schmidt *et al.*, 2005; Ryan *et al.*, 2006; Christen, 2005; Tamayo *et al.*, 2005; Cohen *et al.*, 2015; Orr *et al.*, 2015). This process requires the presence of Mg^{2+} and Mn^{2+} and is inhibited by Ca^{2+} and Zn^{2+} (Rao *et al.*, 2008). The HD-GYP proteins however, directly break the c-di-GMP into two GTP molecules (Ryan *et al.*, 2006).

One intriguing characteristic of GGDEF and EAL domains is that they are often found in the same polypeptide chain as part of multidomain proteins. This organization is puzzling because it is difficult to understand why the same protein contains two domains with opposite enzymatic activities. Theoretically, two possibilities exist that may explain this apparent contradiction. One scenario is that while both domains are enzymatically active, they are differentially regulated by environmental and/or intracellular signals so that at any given point one activity is prevalent. The second possibility is that one of the two domains is enzymatically inactive and it has evolved to carry out new functions. One of these functions may involve binding (but not processing) to the substrate and participation in protein-protein or protein-RNA interactions (Christen, 2005; Ferreira *et al.*, 2008; Romling *et al.*, 2013; Kuchma *et al.*, 2007).

Cellular effectors of c-di-GMP

Although there is breadth of knowledge about c-di-GMP-metabolizing enzymes, we know relatively little about c-di-GMP receptors and targets regulated by c-di-GMP. This is so because enzymes involved in c-di-GMP synthesis and breakdown are readily identifiable due to the characteristic GGDEF, EAL, and HD-GYP domains. In contrast, identifying proteins that function as c-di-GMP receptors/effectors is more challenging because c-di-GMP binds to diverse classes of proteins and other molecules, many of which have no sequence or structural similarity to each other. At present, there are thousands of proteins known to be involved in the synthesis and degradation of c-di-GMP, but only few dozens of receptors have been identified, which are summarized in Table 4.

Table 4. C-di-GMP bacterial receptors

Cellular receptor	Binding motif	Examples	References
PilZ domain proteins	(Q/E) RRxxxR (DZxxG)	YcgR, BcsA, MrKH, MapZ, PlzA	(Amikam and Galperin, 2006; Ryjenkov *et al.*, 2006; Pratt *et al.*, 2007; Freedman *et al.*, 2010; Wilksch *et al.*, 2011; Linghui Xu *et al.*, 2016)
Enzymatically inactive GGDEF proteins with an intact I site	RxxD	PelD, PopA,	(Lee *et al.*, 2007; Duerig *et al.*, 2009; Newell *et al.*, 2012)
Enzymatically inactive EAL proteins	QxxxxxR	LapD, FimX, FilP	(Qi *et al.*, 2011; Newell *et al.*, 2012; Yang *et al.*, 2014)
Transcriptional regulators	Unknown	FleQ, VpsT, Clp, Bcam1349, EspI/Nla24, BldD, BrlR	(Hickman and Harwood, 2008; Leduc and Roberts, 2009; Krasteva *et al.*, 2010; Tao *et al.*, 2010; Fazli *et al.*, 2011; Baraquet *et al.*, 2012; Chambers *et al.*, 2014; Tschowri *et al.*, 2014)
Polynucleotide phosphorylase	Unknown	PnP	(Tuckerman *et al.*, 2011)
GIL domain	RxGD	BcsE	(Xin Fang *et al.*, 2014)
ATPases	GxxxxGKT/S	FliI, HrnC, ClpB2, FleQ, MhseE	(Baraquet and Harwood, 2013; Trampari *et al.*, 2015)
Acyl coenzyme A dehydrogenase	Unknown	Bd2924	(Rotem *et al.*, 2015)
YajQ family	Unknown	XC_3703	(An *et al.*, 2014)
Riboswitches		Vc1, Vc2, Bc1, Bc2, Bc3-5, Cdi2_4	(Sudarsan *et al.*, 2008; Bordeleau *et al.*, 2011; Zhou *et al.*, 2016)

Specificity of the signal transduction system mediated by c-di-GMP

c-di-GMP is a small diffusible molecule that serves to integrate environmental cues from multiple sensory inputs and is synthetized by several proteins encoded in the same genome. Because many DGCs and PDEs can modify the levels of c-di-GMP, a question arises as to how the c-di-GMP produced by a specific DGC can specifically transmit the signal without interfering with the c-di-GMP produced by another DGC. This question has been the focus of intense debate but is still lacking a definitive explanation.

There are several mechanisms that might explain c-di-GMP signal specificity:

Differential expression of GGDEF-EAL proteins

One of the mechanisms to achieve specificity involves co-ordinate regulation of the expression of c-di-GMP components, in which c-di-GMP metabolizing enzymes would be expressed under the same conditions as their downstream receptors. Indeed, some c-di-GMP components are known to be under the transcriptional control of various signals (Weber *et al.*, 2006; Pesavento *et al.*, 2008). However, in *Salmonella*, a genome wide analysis of the expression of the twelve GGDEF domain proteins present in this bacteria showed that almost all genes encoding GGDEF proteins appear to be constitutively expressed in all conditions tested, except for *sen3222*, *yfeA*, *sen2484* and *adrA* (Solano *et al.*, 2009). In the case of *sen3222*, *yfeA* and *sen2484*, protein levels were not detectable in any of the conditions analyzed and in the case of the *adrA* gene, its expression was induced over time, being highly expressed after 72 h of growth in LB media, when a robust biofilm is clearly visible. Thus, this mechanism does not adequately account for *Salmonella* signalling specificity. Accordingly,

(Sommerfeldt *et al.*, 2009) showed that out of a total of 28 GGDEF/EAL protein encoding genes present in *E. coli*, twenty-one are expressed in Luria–Bertani medium, with 15 being under σ^S control.

Co-localization of GGDEF proteins with their receptors:

Another proposed model for signaling specificity involves the spatial sequestration of individual c-di-GMP components, such that the generation and degradation of c-di-GMP would act locally on a particular target. There is some evidence to support local activity of c-di-GMP metabolizing enzymes in some organisms (Merritt *et al.*, 2010; Massie *et al.*, 2012). In *Caulobacter crescentus*, each cellular division results in a mother cell that is non-motile and remains attached to the abiotic surface via an adhesive structure called the stalk, and a daughter cell that is free-living and flagellated. Cell division is regulated by c-di-GMP, the DGC PleD and the PDE TipF that are directed to, and are active at different cell poles in a cell cycle-dependent manner. Progression of the cell cycle is also dependent on the GGDEF I site-type c-di-GMP effector protein PopA. This protein exerts its function allowing initiation of DNA replication through binding to the c-di-GMP produced by PleD in the stalked cell (Christen *et al.*, 2010). Although this kind of regulation may exist in particular cases, the rapid diffusion of c-di-GMP in the bacterial cytoplasm would make it difficult to establish such local pools inside of the bacterial cell in the absence of some kind of compartmentalizing structure (Mills *et al.*, 2011) and there is no firm evidence that such microdomains exist in bacteria.

Affinity of the c-di-GMP by their receptors:

Another mechanism, not mutually exclusive with the previous ones, by which c-di-GMP signalling specificity could be achieved, is through the

binding affinities of downstream receptors. In this scenario, the different GGDEF-EAL proteins of the cell would contribute to a common c-di-GMP cytoplasmic "pool", and the receptor affinity for c-di-GMP would determine which receptors are activated at a particular cytoplasmic c-di-GMP level (Mills *et al.*, 2011).

Like most enterobacteria, *S.* Typhimurium has two known c-di-GMP receptors that contain a PilZ domain: YcgR, which controls flagellar-based motility, and BcsA, the bacterial cellulose synthase. The group of Samuel Miller (Pultz *et al.*, 2012) used a FRET-based method to compare the affinities of the two PilZ domain proteins (YcgR and BcsA) in *Salmonella* and found that they differ by 43-fold. They also demonstrated that when cellular c-di-GMP levels are kept low, neither PilZ domain protein is bound to c-di-GMP, resulting in a motile cell that does not produce cellulose. As cellular DGCs increase the concentration of c-di-GMP past the K_d for YcgR, YcgR becomes bound to c-di-GMP and thus inhibits motility, even at levels of c-di-GMP that are not high enough to bind BcsA. Activation of AdrA expression results in enough c-di-GMP produced to bind to BcsA, and cellulose synthesis occurs. These data greatly supported the hypothesis that regulation by binding affinity of downstream receptors is a mechanism for the selective activation of c-di-GMP controlled processes.

Overview of the c-di-GMP signal transduction system in *Salmonella*

The *Salmonella* genome encodes 20 proteins that may be involved in c-di-GMP synthesis and/or degradation. Five of them are proteins that contain a GGDEF domain, eight contain an EAL domain and seven harbour both GGDEF and EAL domains (Fig. 10). Curiously, this bacterium does not encode any HD-GYP domain protein. Amongst the twelve proteins containing a GGDEF domain, eight show a conserved GGDEF motif,

suggesting that they may act as active diguanylate cyclases. All twelve proteins, with the exception of YciR harbor transmembrane domains and most of them also contain putative sensing domains (Fig. 10). Amongst the fifteen proteins containing an EAL domain, and according to conserved residues in such domain (Fig. 10), eleven may act as active phosphodiesterases.

With respect to the environmental signals that control c-di-GMP synthesis and degradation in *Salmonella*, the group of Samuel I. Miller performed an outstanding study through the use of a biosensor that allowed the screening for signals that altered c-di-GMP concentrations (Mills *et al.*, 2015). Results showed that *Salmonella* senses several compounds, including glucose, N-acetyl-D-glucosamine (GlcNAc), salicylic acid, and specifically L-arginine, among the 20 common amino acids, and responds by rapidly altering the amount of free c-di-GMP.

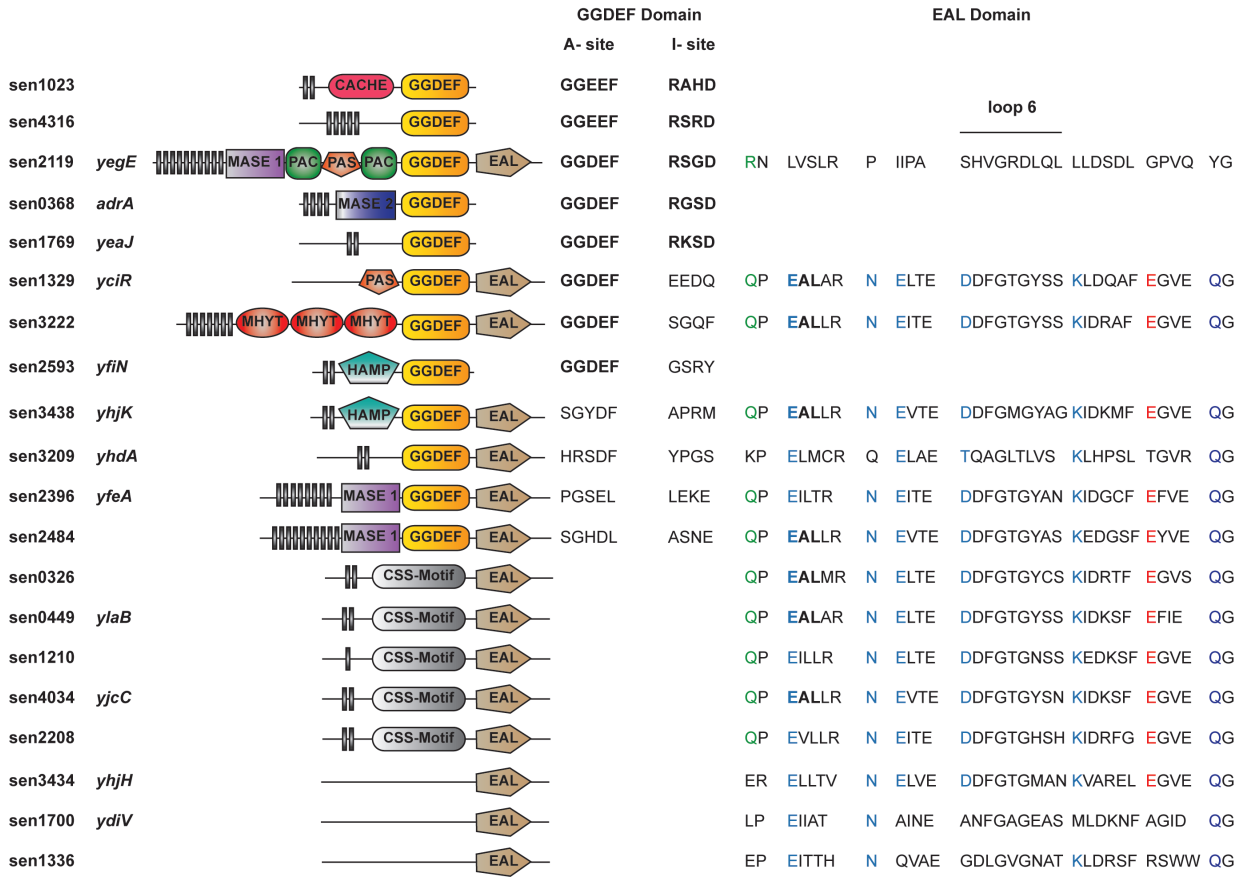


Fig. 10. Summary of the *Salmonella* c-di-GMP metabolyzing proteins. The first column shows the genome position of each gene encoding for a c-di-GMP metabolizing protein. The second column shows the names of currently described genes. Organization of recognized domains by the pfam program (http://pfam.xfam.org/) is displayed through a graphic representation of each protein. The aminoacid sequence of the catalytic A site and the I site of the GGDEF domain is then shown. Conserved aminoacids in the EAL domain are highlighted as follows: residues involved in Mg^{2+} binding (blue); aminoacids that bind c-di-GMP (green); glutamate needed for c-di-GMP hydrolysis (red); loop 6 containing the extended conserved motif DDFG(T/A)GYSS that plays a determinative role in oligomerization of subunits, c-di-GMP binding, and catalysis is underlined. Canonical GGDEF and EAL sites appear in bold.

As it also happens in other bacteria, the large number of enzymes involved in c-di-GMP metabolism in *Salmonella* contrasts with only a handful of c-di-GMP receptors thus far identified (some of them have been inferred from studies performed in *E. coli*). These are (i) YcgR, that binds c-di-GMP at a relatively high affinity (Christen *et al.*, 2007) and inhibits flagellar motility by binding to the motor–stator complex and modulating its function (Boehm *et al.*, 2010; Paul *et al.*, 2010), (ii) BcsA (Ross *et al.*, 1987; Ryjenkov *et al.*, 2006), a c-di-GMP-dependent glycosyltransferase responsible for cellulose synthesis, (iii) PnpA, a 3′-polyribonucleotide polymerase and a 3′-to-5′ exoribonuclease involved in RNA degradation (Tuckerman *et al.*, 2011), (iv) YciR, an unusual PDE that also functions as a c-di-GMP receptor (Lindenberg *et al.*, 2013) and (v) BcsE, a protein encoded in the cellulose synthase operon that is involved in maximal cellulose synthesis (Fang *et al.*, 2014).

With the objective of analyzing the impact of c-di-GMP on *Salmonella* biology, a *Salmonella* strain in which all genes encoding for putative DGCs were deleted was created in our laboratory (Solano *et al.*, 2009). The resulting strain (ΔXII) that was incapable of producing c-di-GMP was therefore unable to produce cellulose. Taken this strain as a basis, a collection of 12 strains, derivatives of ΔXII, each of which contained the chromosomal copy of a single gene encoding a GGDEF-domain protein in the original wild-type genomic location was generated. Phenotypic analysis of this collection of strains revealed that four GGDEF proteins AdrA, SEN1023, YegE and YfiN were individually able to activate cellulose production, supporting the hypothesis that c-di-GMP produced by different GGDEF-domain proteins is able to activate the same biological process and therefore, supporting the model of c-di-GMP specificity based on the affinity of c-di-GMP by their receptors. Virulence assays carried out with ΔXII showed that this strain was highly attenuated in orally and intraperitoneally

infected BALB/c mice. However, further work with ΔXII revealed that it contained an additional chromosomal deletion that included the *rpoS* gene. Since RpoS, which is the master sigma factor during stationary phase and under a variety of stress conditions, plays a critical role in *Salmonella* virulence, the involvement of c-di-GMP in virulence remained unclear. To decipher this and other questions related to c-di-GMP signaling, a new ΔXII strain was again generated in our laboratory, sequenced and confirmed to only lack DGC encoding genes (Zorraquino *et al.*, 2013). This new ΔXII strain has been used throughout this doctoral thesis in order to analyze c-di-GMP influence on *Salmonella* virulence and immunity of the host and also as a genetic tool to manipulate the source and levels of c-di-GMP.

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OBJECTIVES

OBJECTIVES

We have used *Salmonella* Enteritidis as a model organism to analyse the role of c-di-GMP signalling in pathogenicity and also to evaluate the impact of the conserved biofilm exopolysaccharide, PGA, on bacterial biology.

The specific objectives of this thesis are:

1.- Analysis of the role of c-di-GMP signaling pathway in *Salmonella* virulence and evaluation of a strain lacking c-di-GMP and RpoS as a live oral vaccine.

2.- Assessment of the implication of c-di-GMP recognition by its eukaryotic receptor, STING, in the *Salmonella* infection process.

3.- Evaluation of the impact of PGA synthesis on *Salmonella* biology with the aim of elucidating the reasons behind the absence of PGA encoding genes in this bacterial pathogen.

CHAPTER I

Evaluation of a *Salmonella* strain lacking the secondary messenger c-di-GMP and RpoS as a live oral vaccine

SUMMARY

Salmonellosis is one of the most important bacterial zoonotic diseases transmitted through the consumption of contaminated food, with chicken and pig related products being key reservoirs of infection. Although numerous studies on animal vaccination have been performed in order to reduce *Salmonella* prevalence, there is still a need for an ideal vaccine. Here, with the aim of constructing a novel live attenuated *Salmonella* vaccine candidate, we firstly analyzed the impact of the absence of cyclic-di-GMP (c-di-GMP) in *Salmonella* virulence. C-di-GMP is an intracellular second messenger that controls a wide range of bacterial processes, including biofilm formation and synthesis of virulence factors, and also modulates the host innate immune response. Our results showed that a *Salmonella* multiple mutant in the twelve genes encoding diguanylate cyclase proteins that, as a consequence, cannot synthesize c-di-GMP, presents a moderate attenuation in a systemic murine infection model. An additional mutation of the *rpoS* gene resulted in a synergic attenuating effect that led to a highly attenuated strain, referred to as ΔXIII, immunogenic enough to protect mice against a lethal oral challenge of a *S.* Typhimurium virulent strain. ΔXIII immunogenicity relied on activation of both antibody and cell mediated immune responses characterized by the production of opsonizing antibodies and the induction of significant levels of IFN-γ, TNF-α, IL-2, IL-17 and IL-10. ΔXIII was unable to form a biofilm and did not survive under desiccation conditions, indicating that it could be easily eliminated from the environment. Moreover, ΔXIII shows DIVA features that allow differentiation of infected and vaccinated animals. Altogether, these results show ΔXIII as a safe and effective live DIVA vaccine.

INTRODUCTION

Salmonella remains a foodborne pathogen of rising concern to consumers and governments worldwide. In Europe, *Salmonella* is the second most frequently reported cause of foodborne outbreaks with known origin, with *Salmonella enterica* sv *enteritidis* (S. Enteridis) and *Salmonella enterica* sv *typhimurium* (S. Typhimurium) being the two most commonly detected serovars. The number of officially reported clinical cases of salmonellosis amounts to almost 90.000 according to the report of the European Food Safety Authority (EFSA) for the year 2014 (https://www.efsa.europa.eu/en/efsajournal/pub/4329), and the overall economic burden has been estimated to be as high as 3 billion euros a year.

The fact that *Salmonella* gastroenteritis cases usually follow the consumption of contaminated basic food products such as poultry derivatives or pig meat, in combination with the rapid spread of multidrug resistant *Salmonella* spp. triggered by the high-productivity-focused model of animal breeding (González-Zorn and Escudero, 2012; Doyle, 2015) has prompted the appearance of new policies aimed at the prevention of *Salmonella* intake into the food chain. Thus, public health programs including means such as the improvement of hygienic conditions in farms, the use of fodder supplements and the execution of effective vaccination protocols are being gradually implemented. Many *Salmonella* vaccines have been tested in poultry and swine, with varying degrees of success (for review, see (Denagamage *et al.*, 2007; Arguello *et al.*, 2012; Desin *et al.*, 2013)). These can be divided into three categories: live-attenuated, inactivated and subunit vaccines. As regards whole cell killed and subcellular vaccines, biosafety for both human and farm animals is their main advantage. However, it is generally accepted that protection conferred by these last preparations is fairly modest when they are compared to

vaccines based on live attenuated *Salmonella* strains (Mastroeni *et al.*, 1993; Simon *et al.*, 2011). This assertion is supported by the potential of live bacteria to activate both humoral and cellular adaptive immune responses (Mastroeni and Ménager, 2003) and by their capacity to inhibit intestinal colonization during the "immunity gap" (period of time after neonatal vaccination when there is no longer sufficient maternal immunoglobulins to afford protection from infection but when there is still enough of this maternal protection to prevent the young animal from mounting its own protective immune response) (Methner *et al.*, 2011). If other evidences like the easiness of production and administration are considered, we obtain a scenario in which the livestock industry calls for new live attenuated vaccines that display an improved balance between attenuation (safety) and immunogenicity (efficacy).

Since the early 90´s, attenuation of *Salmonella* has been accomplished by the introduction of mutations in *aro* genes and global regulators like PhoPQ, Crp or RpoS (for review, see (Karasova *et al.*, 2009)). Other common attenuation strategies are based on auxotrophies raised by mutation of genes involved in the synthesis of purines (*purA*, *purE* mutants) or in the metabolism of carbohydrates (*galE* mutants) (O'Callaghan *et al.*, 1990; McKenna *et al.*, 1995) and on the elimination of determinants directly involved in infection establishment (e.g. pathogenicity islands, virulence plasmid, *svp* genes) (Bohez *et al.*, 2008; Matulova *et al.*, 2012). Present-day advances widening the knowledge about molecular mechanisms underlying *Salmonella* virulence and the development of novel DNA engineering tools are currently leading to more ambitious genetic approaches in the area of recombinant vaccines, and long-term visions include *Salmonella* strains with regulated delayed attenuation *in vivo* and their use as antigen carrier/delivery platforms (Kong *et al.*, 2012). Nevertheless, fairly curious is the fact that some of the strains used as

commercial vaccines, such as SG 9R, VacT or VacE, have been generated by passages in the laboratory or by random mutagenesis and thus, the exact genetic basis of their attenuation is largely unknown (Karasova *et al.*, 2009).

In our laboratory, we constructed a *S.* Enteritidis strain, called ΔXII, carrying mutations in the twelve genes encoding GGDEF domain proteins and thus, incapable of synthesizing the secondary messenger bis-(3–5)-cyclic dimeric GMP (c-di-GMP) (Solano *et al.*, 2009). This molecule is widely recognized as a key regulator of bacterial biology, including the transition from a planktonic to a biofilm lifestyle and from the virulent state in acute infections to a less virulent state characteristic of chronicity (Römling *et al.*, 2013). Furthermore, bacterial cyclic nucleotides have been recently involved in modulating the innate immune response, leading to induction of type I interferons, via direct binding to the eukaryotic Stimulator of Interferon Genes (STING) and to DDX41 (Woodward *et al.*, 2010; Burdette *et al.*, 2011; Parvatiyar *et al.*, 2012; Valle *et al.*, 2013). Virulence assays with *S.* Enteritidis ΔXII showed that it was highly attenuated in orally and intraperitoneally infected BALB/c mice (Solano *et al.*, 2009) and hence, this strain might be considered a potential vaccine candidate. However, further work with ΔXII revealed that it contained an additional chromosomal deletion of 16.8 kilobases that included the *rpoS* gene (Zorraquino *et al.*, 2013). Since RpoS, which is the master sigma factor during stationary phase and under a variety of stress conditions, plays a critical role in *Salmonella* virulence (Fang *et al.*, 1992), it was unclear whether the absence of c-di-GMP played any role in virulence attenuation shown by ΔXII. To solve this and other questions related to c-di-GMP signaling, a new ΔXII strain was generated, sequenced and confirmed to only lack GGDEF proteins encoding genes (Zorraquino *et al.*, 2013).

Taking new ΔXII strain as a basis, the present work was planned with three main objectives: (i) analyzing the contribution to *Salmonella* virulence of c-di-GMP signaling; (ii) confirming the attenuation of a strain deficient in c-di-GMP signaling and RpoS with the aim of identifying a genetic background that might lead to a novel attenuated *Salmonella* vaccine strain and, (ii) assessing its potential use as an effective vaccine to combat gut and organ colonization by *Salmonellae*. Here, we report that total depletion of c-di-GMP in new ΔXII correlated with a moderate loss of virulence in a murine model of infection. A ΔXII strain in which we also performed a *rpoS* mutation led to a strain that we called ΔXIII, more attenuated than a single *rpoS* mutant but still capable of eliciting a cellular/humoral balanced, long-lasting immune response against a lethal oral-challenge of a *S*. Typhimurium virulent strain. Moreover, use of ΔXIII enabled serological differentiation of vaccinated and infected mice due to the lack of antibodies raised against the *Salmonella* specific GGDEF protein SEN4316 in vaccinated animals.

MATERIALS AND METHODS

Ethics statement

Animal studies were performed in accordance with the European Community guiding in the care and use of animals (Directive 2010/63/EU). Protocols were approved by the ethics committee of the Public University of Navarra (Comité de Ética, Experimentación Animal y Bioseguridad of the Universidad Pública de Navarra) (approved protocol PI-004/11). Work was carried out in the animal facility of the Instituto de Agrobiotecnología, Universidad Pública de Navarra. Animals were housed under controlled environmental conditions with food and water ad libitum. Mice were euthanized by CO_2 inhalation followed by cervical dislocation and all efforts were made to minimize suffering.

Bacterial strains and culture conditions

Bacteria were grown in LB broth and on LB agar with appropriate antibiotics at the following concentrations: kanamycin (Km), 50 μg ml^{-1}; ampicillin (Am), 100 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1} and apramycin (Apr), 60 μg ml^{-1}.

Bacterial strains used in this study are listed in Table 1. *S.* Enteritidis 3934 (MIC-54) is a wild type clinical isolate described in (Solano *et al.*, 1998; Solano *et al.*, 2002). *S.* Enteritidis ΔXII (MIC-1324) is a multiple mutant, derivative of 3934, carrying mutations in all genes encoding GGDEF domain proteins and thus incapable of synthesizing c-di-GMP (Zorraquino *et al.*, 2013). This multiple mutant was generated by a markerless gene replacement method described in (Solano *et al.*, 2009; Zorraquino *et al.*, 2013) and as a consequence, does not contain any exogenous DNA. To compare the virulence of the wild type and ΔXII strain we used a ΔXII derivative (MIC-3664), resistant to kanamycin and tetracycline, that was generated in (Zorraquino *et al.*, 2013).

S. Typhimurium CNM 143/09 (MIC-1201) is a clinical strain isolated from a patient that suffered a pig meat borne gastroenteritis (Centro Nacional de Microbiología; Instituto de Salud Carlos III).

To generate the single mutant Δ*rpoS* (MIC-2101), a PCR-generated linear DNA fragment in combination with the helper plasmid pKOBEGA were used (Chaveroche *et al.*, 2000; Solano *et al.*, 2002). A selectable antibiotic resistance gene was generated by PCR using oligonucleotides rpoS-Apra_fw (ctgaaagttcatgatttaaatgaagacgcggaatttgatgagaacggagtagaggctttttcccatccac cggatcaatt) and rpoS-Apra_rv (tgcgctcgttgagacgaagcatacggctgacgtcatcaaccggtttatccagttgctctgtccttgttagac attatttg) that included 60-nt homology extensions for the targeted locus and 20-nt priming sequences for the apramycin resistance gene as template from a freshly isolated colony of *E. coli* MC4100 F´tet Δ*traD*::*aac* (García *et al.*, 2004). Electroporation (25 μF, 200 Ω, 2.5 kV) was carried out according to the manufacturer's instructions (Bio-Rad) using 50 μl of cells and 1 to 5 μg of purified and dialyzed (0.025 μm nitrocellulose filters; Millipore) PCR product. Shocked cells were added to 1 ml of LB broth, incubated overnight at 30°C, and then spread on LB agar with Apr to select Apr^R transformants after incubation at 30°C for 24 h. Mutants were then grown on LB broth with Apr at 43°C for 24 h and incubated overnight on LB agar with Am at 30°C to test for the loss of the helper plasmid.

To generate the vaccine candidate, *S.* Enteritidis ΔXIII (MIC-1330), the *rpoS* gene was deleted in ΔXII (MIC-1324) strain as follows. DNA fragments corresponding to the upstream (fragment AB) and downstream (fragment CD) regions of the *rpoS* gene were amplified with the following oligonucleotides, with chromosomal DNA from strain *S.* Enteritidis 3934 as a template. Primers RpoS_1´_Fw (gaatcgtatacaatcgccag) and RpoS_B_XhoI (ctcgaggctcctacccgtgatcc) were used to amplify the AB fragment. Primers

RpoS_C_XhoI (ctcgagttgtcaaaaaaaggccagtc) and RpoS_D_BglII (agatctaatctgccacaggtgatg) were used to amplify the CD fragment. The PCR products were cloned in the pGEMt-easy vector (Promega), digested with NotI and XhoI enzymes in the case of the AB fragment and XhoI and BglII enzymes in the case of the CD fragment, and ligated in the same ligation mixture with the pKO3blue vector (Solano *et al.*, 2009) digested with NotI and BglII enzymes. The recombinant pKO3blue::AD vector was extracted from *E. coli* XL1 Blue and electroporated into strain *S.* Enteritidis ΔXII. The following steps of integration and excision of the plasmid were performed as described previously (Solano *et al.*, 2009). As a result, ΔXIII is a multiple mutant in all genes encoding GGDEF domain proteins and in *rpoS* and does not contain any exogenous DNA.

Mice survival assays

Mice were acclimated for 7 days after arrival before the experiments were started. In the case of oral infections, food and water were removed, twelve and two hours respectively, before the administration of bacterial suspension. Mice were prefed with 20µl of 10% sodium bicarbonate 30 min before bacterial inoculation. Water and food were again supplied right after inoculation.

For mice survival assays, eight-week-old female BALB/c mice (Charles River Laboratories) were infected orally with 10^7 *Salmonella* wild type or mutant cells that had been grown overnight in LB broth and resuspended in 100 µl of PBS. Mice survival was monitored for 20-28 days. Over the course of infection, mice were examined twice per day and a final disease score was given to each mouse according to clinical signs observed as follows. No clinical signs (0); mild clinical signs: ruffled fur (1); moderate clinical signs: ruffled fur plus, lethargy, hunched posture and decreased activity (2); severe clinical signs: paresis, paralysis, tremor, shivers, ataxia, rigidity (3).

When evident signs of disease (score 2 to 3) were observed, mice were euthanized by CO_2 inhalation followed by cervical dislocation.

Mice competitive infections

Infection studies were carried out with 8-week-old female BALB/c mice (Charles River Laboratories). Mice were inoculated intraperitoneally (i.p.) or intragastrically (i.g.) with 100 µl of bacteria suspended in phosphate-buffered saline (PBS; pH 7.4). The total bacteria inoculum was $2x10^4$ cfu (i.p.) or $2x10^8$ cfu (i.g.) of combined *S.* Enteritidis wild-type and mutant strains at a ratio of 1:1. The cfu of each strain in the inoculum (input) was quantified by plating dilution series on LB agar and using antibiotic resistance to distinguish between strains. Mice were euthanized after 3 days (i.p.) or 5 days (i.g.), and dilution series of liver and spleen lysates were plated on LB agar for enumeration of cfu (output), using antibiotic resistance to differentiate strains. Values for competitive index (CI) were calculated as the ratio of wild type to mutant in the output divided by that in the input, and the CI was expressed as $\log_{10}$ (Figueira *et al.*, 2013).

To compare the *in vivo* interaction of *Salmonella* strains with murine intestinal epithelial cells, the ligated ileal loop co-infection model was used as described previously (Solano *et al.*, 2002). Values for CI were calculated as described above.

Mice protection assay

Groups of seven 8-week-old female BALB/c mice were orally inoculated with 10^7 cfu of the attenuated strains ΔXIII or ΔrpoS. One control group only received PBS. On day thirty-three after immunization, immunized and control animals were intragastrically challenged with 10^6 cfu of virulent *S.* Typhimurium 143/09. Vaccinated and control mice were monitored daily to assess clinical signs as described above. When evident signs of disease

(score 2 to 3) were observed, mice were opportunely euthanized by CO_2 inhalation followed by cervical dislocation.

Preparation of ΔXIII heat-killed lysate

The vaccine strain, ΔXIII, was cultured overnight at 37°C on LB agar plates. Bacteria were resuspended in PBS and the OD_{600} was adjusted to 0.2 ($1x10^8$ cfu ml^{-1}, based on viable cell counts on LB agar). Bacteria were then incubated at 75°C in a water bath for 30 min. The effectiveness of the inactivation was assessed by plating 100 µl of the suspension on LB agar, after treatment.

Determination of antibody titers

Blood samples from the orbital vein of each mouse from the control and the ΔXIII-immunized groups were collected before immunization and 14 and 28 days after immunization. Samples were centrifuged and sera from each group were collected. Serum IgG and IgM were detected by ELISA. For that, Nunc Maxisorp 96-well plates (Thermo Fisher Scientific, San Jose, CA) were coated with 10^7 cfu of heat-killed ΔXIII bacteria and incubated at 4°C overnight. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T; pH 7.4) and blocked with 5% nonfat dried milk powder in PBS-T at room temperature for 1 h. After three washes with PBS-T, immune mouse sera were serially diluted from an initial dilution of 1:20. A 100 µl volume of diluted sample was added to duplicate wells and incubated at 4ºC overnight. Wells were washed three times with PBS-T and 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgM (H+L) secondary antibody diluted 1:2500 (Thermo Scientific) was added to each well. The plates were incubated for 1 h at room temperature and then washed three times. One hundred microliters of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) were added to each well and

the absorbance at 405 nm was determined on an ELISA reader. Endpoint titers were expressed as the reciprocal $\log_2$ values of the last positive sample dilution. Absorbance readings higher than the mean plus two standard deviations of preimmune serum baseline values were considered indicative of positive reactions.

Opsonophagocytosis uptake assay

The murine macrophage-like cell line J774.2 was grown in RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomicin (Lonza) at 37ºC with 5% CO_2. Cells were seeded in 24-well tissue culture plates. Once in confluence (0.2 x 10^6 cells per well), cells were washed with PBS, and medium without FBS was added 16 hours prior to the experiment. Fourteen hours later, the medium was replaced by medium without antibiotics. 2 x 10^6 cfu of an overnight culture of *S.* Typhimurium143/09 was mixed with 10% of pooled immune serum, pre-immune serum or PBS in 500 µl of Minimum Essential Medium Eagle (MEM) with 1% Bovine Serum Albumin (BSA) and complement (complement sera rabbit, Sigma-Aldrich). After incubation at room temperature for 15 minutes, the mix was added to the monolayer cells (multiplicity of infection of 10) and plates were incubated for 30 minutes at 37ºC in 5% CO_2. To kill extracellular bacteria, cells were washed and incubated for 90 minutes with 500 µl of RPMI 1640 containing 100 µg/ml gentamicin (Sigma-Aldrich). Cells were washed three times with sterile PBS and lysed with 0.1% Triton X-100. The number of phagocytosed bacteria was determined by plating serial dilutions of the lysates on LB agar and data were represented as relative bacterial uptake of bacteria mixed with serum with respect to the uptake of bacteria suspended in PBS, which was given a value of 1.0. Experiments were conducted in triplicate.

ELISPOT assay

The number of IFN-γ producing cells was measured by ELISPOT using a kit from BD-Biosciences (BD Biosciences, San Diego, CA) according to the manufacturer´s instructions. Splenocytes were harvested from individual mice 28 days after immunization. Ninety-six-well Multi-Screen high protein binding Immobilon-P membrane plates (Millipore, Billerica, MA) were coated with mouse anti–IFN-γ capture antibody, incubated overnight, and blocked for 2 h with RPMI containing 10% fetal calf serum. Then, 1 x 10^6 splenic cells in 10% fetal calf serum-RPMI were added to each well in triplicate and cultured in the presence or in the absence of 10^7 cfu of heat-killed ΔXIII bacteria. Following overnight incubation, the plates were washed three times with PBS and incubated with biotinylated anti-IFN-γ antibody for two hours. Wells were washed three times and incubated with a solution of streptavidin-peroxidase for one hour. Then, plates were washed and developed with freshly prepared 3,3′-Diaminobenzidine (DAB) solution. The reaction was stopped with distilled water and spots were counted using an ELISPOT automated reader (CTL; Aalen, Germany).

Measurement of cytokine production by ELISA

1 x 10^6 splenic cells harvested from individual mice 28 days after immunization were co-cultured with 10^7 cfu of heat-killed ΔXIII bacteria during 48 h. RPMI media was used as negative control. IL-2, IL-10 and IL-17 released to the supernatant were measured by ELISA using specific kits from BD Biosciences (San Diego, CA) according to the manufacturer´s instructions.

Levels of IFN-γ and IL-5 in serum were determined using a Mouse Procarta Cytokine 2Plex Assay (eBioscience). Levels of IL-10 in serum were determined with a Mouse IL-10 ELISA Ready-SET-Go!® kit (eBioscience).

Flow cytometry analysis

Splenocytes (2x106/well) harvested from individual mice 28 days after immunization were either stimulated with 106 and 107 cfu of heat-killed ΔXIII bacteria or rested unstimulated for 1h. Golgi-Plug and Golgi-Stop (BD Biosciences, San Diego, CA) were added and plates were incubated for five hours. Then, cells were washed with staining buffer (PBS, 0.5% BSA, 2 mM EDTA), surface stained with anti-CD8a-APC (53-6-7) and anti-CD4-FITC (RM45) monoclonal antibodies (mAbs), fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and finally stained for intracellular cytokines with anti-TNF-α-PE-Cy7 (MP6-XT22) and anti-IFN-γ-PE (XMG1,2) mAbs. All mAbs were from Biolegend (San Diego, CA). Dead cells were excluded by FSC and SSC. Data were collected in a FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA).

Biofilm formation and desiccation experiments

Biofilm formation in a rich-medium condition (LB) was determined and visualized as previously described (Solano *et al.*, 2002). Colony morphology and color on Congo red agar plates were tested after 48 hours of incubation at 28 °C (Romling *et al.*, 1998).

The desiccation experiment was adapted from a described protocol (White *et al.*, 2006). Briefly, 100 µl from overnight cultures grown in LB medium at 37 °C were tested immediately (initial numbers) or air dried and stored in 24 well tissue culture plates at room temperature for 12 days. After

rehydration of bacteria in 500 µl PBS, pH 7.4, the number of viable cells remaining in each sample was determined by serially diluting cell mixtures and plating in duplicate. The experiment was conducted in triplicate.

Production of recombinant SEN4316

The *sen4316* gene was amplified from wild type genomic DNA with primers sen4316 BamHI-fw (ggatccatgacaacaccatcctggcg) and sen4316 SalI-rv (gtcgactcatagggcgcgcatgtcgt), using Phusion High-Fidelity DNA Polymerase (Fermentas-Thermo Scientific). The PCR-amplified fragment was cloned in pGEM-T Easy (Promega), sequenced and digested with BamHI and SalI to clone it into the pET28a vector (Novagen). The resulting plasmid pET28a::*sen4316* was electroporated into *E. coli* BL21 C43 (DE3) (Miroux and Walker, 1996). Cultures were grown at 37°C, 250 rpm, to an optical density (OD_{600}) of 0.5, and isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM. Cells were then grown overnight at 23°C. Harvested cells were lysed with BugBuster HT Protein Extraction Reagent (Merck Millipore). SEN4316 accumulated in inclusion bodies was obtained by centrifugation and suspension of insoluble material in CTAB 1%, incubation at room temperature for 1 h and recovery of the supernatant by centrifugation at 20,000 x g. This supernatant was dialyzed against binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) and the recombinant protein was purified with a His GraviTrap affinity column according to standard protocols (GE Healthcare). Eluted protein was dialyzed against sterile water, analyzed by SDS–PAGE and Western-Blot and lyophilized.

SEN4316 based ELISA

Groups of ten mice were orally inoculated with a single dose of 10^7 cfu or 10^4 cfu of ΔXIII or the wild type strain, respectively. Blood samples from

the orbital vein of each mouse were collected before inoculation and 33 and 44 days after inoculation. Samples were centrifuged and sera from each group were collected and pooled. Nunc Maxisorp 96-well plates (Thermo Fisher Scientific, San Jose, CA) were coated with SEN4316 (2 µg/well) in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were then washed three times with PBS-T and blocked with 5% nonfat dried milk powder in PBS-T at room temperature for 2 h. After three washes with PBS-T, 100 µl of pooled sera diluted 1:100 in PBS-T containing 2.5 % of BSA were added to each well and incubated at room temperature for 2 h. Wells were washed three times with PBS-T and 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgM (H+L) secondary antibody diluted 1:1250 (Thermo Scientific) was added to each well. The plates were incubated for 2 h at room temperature and then washed three times. One hundred microliters of ABTS (Sigma-Aldrich) were added to each well and the absorbance at 420 nm was determined on an ELISA reader.

sen4316 **PCR to differentiate vaccinated from infected mice**

Faecal samples from the wild type and ΔXIII infected groups were pooled and collected at day 1, 7, 14 and 21 post-infection. Cages were cleaned daily to remove non collected faeces. DNA from stool was extracted using a QIAamp DNA Stool Mini Kit (Qiagen). Oligonucleotides DIVA-1 (cacgattacgccaactcgagttgt) and DIVA-2 (gtaagataactgtgcgaag) were used to amplify a 632 bp fragment from ΔXIII DNA. Amplification of *invA* with oligonucleotides invA-fw (ggcgatattggtgtttatgg) and invA-rv (catattatcgctatcgccat) was used to amplify a 658 bp fragment from wild type and ΔXIII DNA. The PCR amplification conditions were as follows: 1 cycle at

95°C for 5 min; 30 cycles of the following: 94°C for 30 s, 54°C for 30 s and 72°C for 35 s; 1 cycle of 72°C for 7 min.

Statistical analyses

All statistical analyses were performed in GraphPad Prism 5.01. A Log-rank (Mantel-Cox) test was used to assess significance in mice survival assays. A Wilcoxon signed-rank test was used to determine whether the CI values were significantly different from the hypothetical value of zero. A two-way analysis of variance combined with the Bonferroni test was used to analyze statistical significance in antibody titers, cytokine serum levels and data from flow cytometry assays. A nonparametric Mann-Whitney test was used to assess significant differences in the opsonization assay. Differences in cytokine production in splenocytes assayed by ELISPOT and ELISA were determined using the unpaired Student's t test.

Table 1. Strains used in this study

Strain	Genotype	Degree of attenuation	MIC[a]	Reference or source
S. Enteritidis				
3934	Wild-type clinical isolate	-	54	(Solano *et al.,* 1998) (Solano *et al*., 2002)
ΔXII	3934 Δ*adrA* Δ*sen1023* Δ*yeaJ* Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* Δ*yfeA* Δ*sen4316*	+	1324	(Zorraquino *et al*., 2013)
ΔXII Km^R Tc^R	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*:: Km^R Δ*sen4316*	+	3664	(Zorraquino *et al*., 2013)
ΔXIII	3934 Δ*adrA* Δ*sen1023* Δ*yeaJ* Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* Δ*yfeA* Δ*sen4316* Δ*rpoS*	+++	1330	This study
Δ*rpoS*	3934 *rpoS*::Apr^R	++	2101	This study
S. Typhimurium				
S. Typhimurium 143/09	Wild-type clinical isolate	-	1201	Centro Nacional de Microbiología
E.coli				
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdr*17 *supE*44 *rel*A1 *lac* [F´*proAB lacI*^{q} Z Δ*M15* Tn10 (Tc^R)]		797	Stratagene
MC4100 Apr^R	MC4100 F´tet ΔtraD::aac		1087	(García *et al*., 2004)
E.coli BL21 C43(DE3)	*huA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*		1637	(Miroux *et al*., 1996)

[a]Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra.

RESULTS

Analysis of the contribution of c-di-GMP signaling to *Salmonella* virulence

To evaluate the impact of the absence of c-di-GMP on *Salmonella* virulence, we firstly compared the survival rates of groups of seven BALB/c mice, which are susceptible to systemic infection with *Salmonella*, orally infected with 10^7 cfu of the wild type *S.* Enteritidis strain 3934 or its derivative, new ΔXII strain. ΔXII showed virulence attenuation that manifested by a latter time to death than animals infected with wild type *Salmonella*. However, the difference between survival curves was no statistically significant (p=0.2287) and at the end of the experiment ten and twenty percent of mice survived after infection with the wild type and ΔXII respectively (Fig. 1A). Virulence of ΔXII after infection by the natural oral route was further investigated by carrying out a competitive index (CI) analysis in an ileal loop coinfection experiment and also, by assessing the level of organ colonization following oral co-inoculation of the wild type and ΔXII strains. ΔXII showed a significantly reduced capacity to adhere and invade the intestinal epithelium (Fig. 1B) and a significantly lower ability to colonize livers and spleens of orally infected mice (Fig. 1C). Defectiveness of ΔXII colonization at systemic sites was additionally confirmed by evaluating organ colonization after intraperitoneal (i.p) infection. ΔXII mutant colonized both the spleen and liver of i.p infected mice significantly less than the wild type strain (Fig. 1D). Taking together, these results showed that c-di-GMP signaling is required for natural virulence of *Salmonella* in BALB/c mice, since absence of c-di-GMP rendered bacteria significantly less able to adhere and invade the intestinal epithelium and to colonize systemic organs during acute infection. However, the overall outcome of the lack of c-di-GMP signaling in pathogenicity is subtle, resulting in a non significant virulence attenuation.

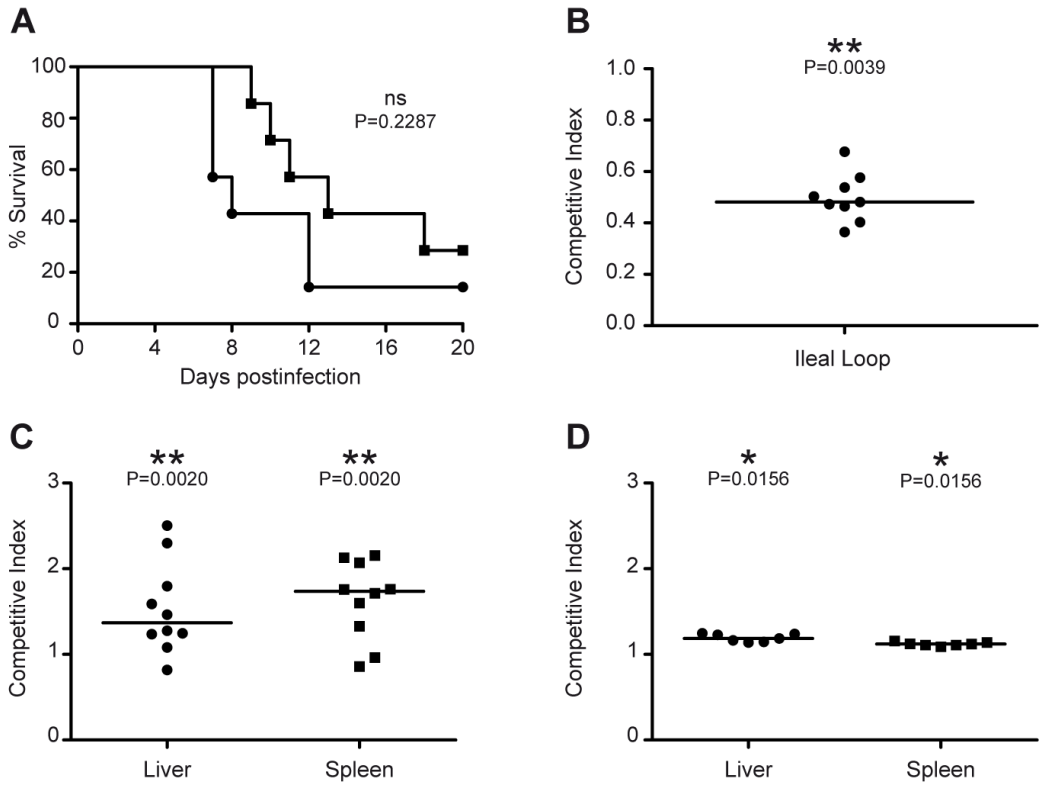

Fig. 1. c-di-GMP signaling in *Salmonella* is required for intestinal epithelium and organ colonization of BALB/c mice but its absence does not significantly affect BALBc mice survival. (A) Comparative lethality between wild-type (black circles) and ΔXII (black squares) strains in an oral infection mouse model. Inoculum administered was 10^7 cfu/mouse. P-value was determined by a Log-rank (Mantel-Cox) test. ns; no significant difference. (B) Competitive index (CI) analysis of wild type and ΔXII strains after performing an ileal loop coinfection experiment. Nine ileal loops were coinfected with $2x10^7$ cfu containing equal numbers of the parental and ΔXII strains. (C) CI analysis following intragastric inoculation of ten BALBc mice with a 1:1 mixture of wild type and ΔXII strains (total inoculum administered was 2 x 10^8 cfu). Mice were sacrificed after five days and bacteria were enumerated from livers and spleens. (D) CI analysis following intraperitoneal inoculation of seven BALBc mice with a 1:1 mixture of wild type and ΔXII strains (total inoculum administered was 2 x 10^4 cfu). Mice were sacrificed three days postinoculation and bacteria were enumerated from livers and spleens. CI was defined as the $\log_{10}$ of the ratio of wild type strain to ΔXII strain recovered (Output) divided by the ratio of wild type strain to ΔXII strain present in the inoculum (Input). A CI > 0 indicates wild type with a colonization advantage compared to ΔXII and a CI < 0 indicates wild type with a colonization disadvantage over ΔXII. The plots display values obtained from individual samples and the median CI is represented by horizontal bars. P-values were determined by a Wilcoxon signed-rank test. *P < 0.05; ** P < 0.01; *** P < 0.001.

Construction and virulence characterization of a *Salmonella* strain lacking all GGDEF proteins and the RpoS sigma factor

Results described above carried out with new ΔXII strain, which only lacks GGDEF proteins encoding genes, demonstrated that absence of c-di-GMP has a restrained impact on virulence. On the other hand, the firstly described ΔXII strain, that also contains a chromosomal deletion that includes the *rpoS* gene, is completely avirulent in mice (Solano *et al.*, 2009). Hence, we next wondered whether this avirulent phenotype was solely caused by the absence of RpoS or by the synergic effect of the lack of RpoS and c-di-GMP. To analyze this, we compared the pathogenic behavior of the wild type strain, a single *rpoS* mutant, and a derivative of new ΔXII strain in which we deleted the *rpoS* gene. This novel mutant was named ΔXIII. A survival experiment of orally infected BALB/c mice with 10^7 cfu of each strain showed that, in agreement with our previous results, ΔXIII strain exhibited a total attenuated behavior and thus, all mice infected with this strain survived at day 28 and did not present any morbid symptoms (Fig. 2A). In contrast, the survival rate of mice infected with the wild type strain was only 10% at the end of the experiment, whereas infection with the *rpoS* mutant rendered a final survival of 60% of infected mice (Fig. 2A). To certify virulence loss of ΔXIII, mice inoculated with this strain were sacrificed at the end of the experiment and a complete clearance of infection was confirmed by the absence of *Salmonella* in livers, spleens and faeces.

Since a *Salmonella* vaccine strain needs to be invasive enough to induce durable immunity in the host and thus, an excessive attenuation of ΔXIII strain was not desirable, we tested its invasiveness capacity at an earlier time of infection. For that, we orally infected groups of seven BALB/c mice with $2x10^7$ cfu of a 1:1 mixture of the wild type and Δ*rpoS* or ΔXIII strains. At day 7 post infection, animals were euthanized, bacterial load in spleens was determined and the CI was calculated. As shown in Fig. 2B,

ΔXIII was capable of reaching and colonizing internal organs, although, when compared to the wild type strain, it presented an approximately 10^4 fold defect in its ability to colonize spleens of infected mice. Again, Δ*rpoS* mutant exhibited an intermediate attenuation phenotype, confirmed by a 10^2 deficiency in spleen colonization.

Altogether, these data show that in ΔXIII strain, the absence of both c-di-GMP signaling and the sigma factor RpoS exerts a synergistic attenuating effect on *Salmonella* virulence, that turns this novel mutant strain into a potential live attenuated vaccine.

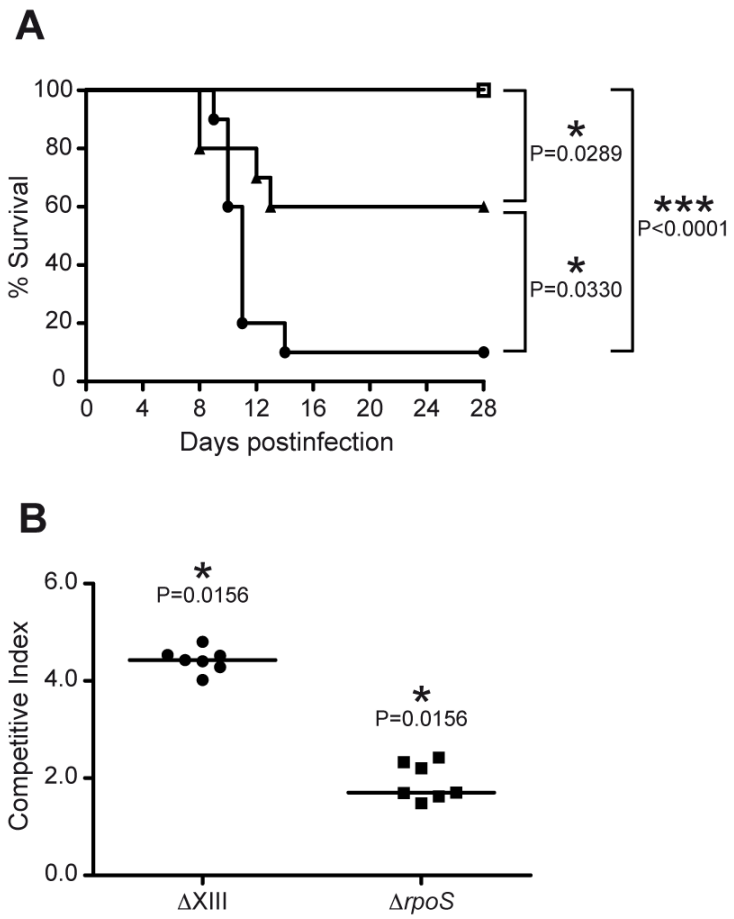

Fig. 2. The synergic effect of the absence of RpoS and c-di-GMP signaling in ΔXIII strain results in high virulence attenuation. (A) Comparative lethality between wild-type (black circles), ΔrpoS (black triangles) and ΔXIII (open squares) strains in an oral infection mouse model. Groups of ten mice were orally infected with 10^7 cfu/mouse. P-values were determined by a Log-rank (Mantel-Cox) test. (B) CI analysis following intragastric inoculation of seven BALBc mice with a 1:1 mixture of wild type and ΔXIII or ΔrpoS strains (total inoculum administered was 2 x 10^7 cfu). Mice were sacrificed after seven days and bacteria were enumerated from spleens. CI for the wild type and mutant strains was defined as stated in Figure 1 legend.

Evaluation of the protection conferred by vaccination with ΔXIII

With the aim of investigating the efficacy of ΔXIII strain as an orally administered vaccine against salmonellosis, a vaccination-challenge analysis was carried out in BALBc mice, using a clinical isolate of *S.* Typhimurium as the challenge strain. The reason why this strain was used for challenge is that, according to the report of the European Food Safety Authority (EFSA) for the year 2014 (https://www.efsa.europa.eu/en/efsajournal/pub/4329), *S.* Enteritidis and *S.* Typhimurium are the two most commonly reported serovars in confirmed human cases. Moreover, *S.* Enteritidis is the second most commonly reported serovar from broiler meat and *S.* Typhimurium is the most frequently reported serovar in pigs and cattle and also in pig meat. By using *S.* Typhimurium for challenge we assessed both ΔXIII vaccination efficacy and also the level of cross-protection against other *Salmonella* serovar. Groups of 7 mice were orally immunized with a single dose of 10^7 cfu of either the ΔXIII or ΔrpoS strain. This last strain was used as an immunizing control. A third group of mice was given 100 µl of sterile PBS. Vaccinated and control untreated mice were challenged 33 days post immunization with 10^6 cfu (10 fold the LD_{50}) of *S*. Typhimurium 143/09 and data on number of dead mice were daily collected. As shown in Fig. 3, all non vaccinated animals died within a 10 day period post challenge. In contrast, 90% of ΔXIII immunized mice survived after challenge. Vaccination with Δ*rpoS* resulted much less effective, firstly because, according to the results presented above, 30% of mice died before challenge, and secondly, because, after challenge, only 30% of initial mice remained alive. These results demonstrated that immunization with ΔXIII confers a very significant degree of crossprotection against salmonellosis by *S.* Typhimurium in mice.

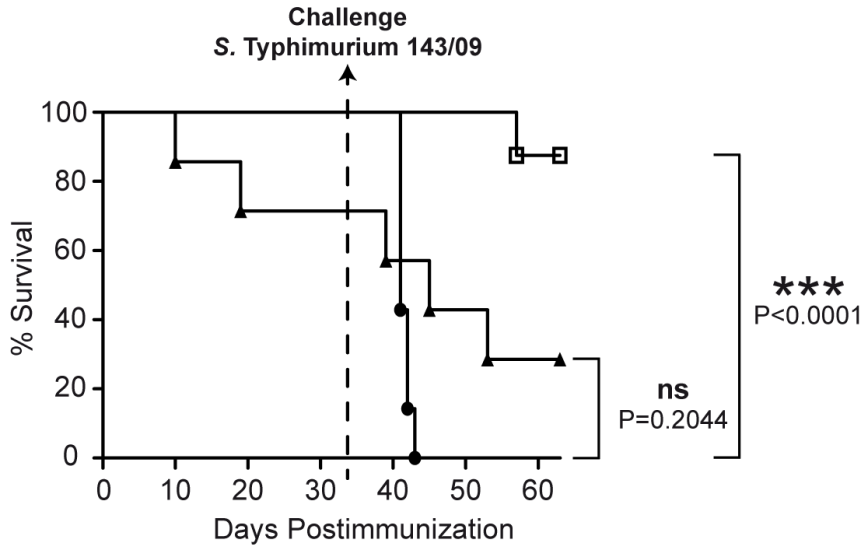

Fig. 3. Vaccination-challenge analysis of the protection conferred by ΔXIII. Groups of seven BALBc mice were orally vaccinated with 10^7 cfu of ΔXIII (open squares), Δ*rpoS* (black triangles) or PBS as a control (black circles). 33 days post immunization, mice were challenged with 10^6 cfu/mouse of *S.* Typhimurium 143/09. Survival curves were plotted and P-values were determined by a Log-rank (Mantel-Cox) test.

Characterization of the immune response generated by immunization with ΔXIII strain

While performing the vaccination experiments described above, and in order to know the basis of the protection conferred by ΔXIII, serum from mice of the control and ΔXIII immunized groups were collected right before immunization and 14 and 28 days after immunization. An ELISA assay was then carried out to measure the levels of IgG and IgM against a ΔXIII heat-killed lysate in individual sera. Results showed a very significant antibody response in mice immunized with ΔXIII strain when compared to control mice, treated with PBS (Fig. 4A). Then, pooled sera samples were used to examine the efficiency of such generated antibodies to promote opsonization and phagocytosis of *S.* Typhimurium 143/09 by J774.2 murine macrophage cells. As shown in Fig. 4B, the number of phagocytized bacteria was significantly higher when bacteria were preincubated with 10% of sera from ΔXIII immunized animals than when incubated with preimmune sera. The specificity of these antibodies against *Salmonella* was confirmed by the fact that the uptake of bacteria preincubated with preimmune sera was very similar to the uptake of bacteria suspended in PBS (relative bacterial uptake close to one).

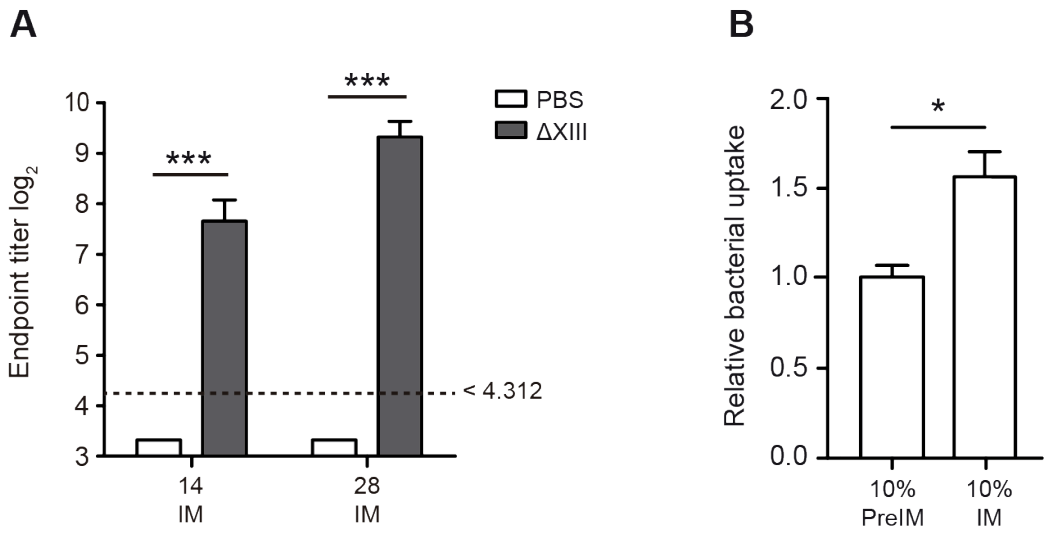

Fig. 4. Humoral immune responses in mice immunized with ΔXIII strain. A) Sera obtained 14 and 28 days post-immunization from mice immunized with ΔXIII present significantly higher levels of IgG and IgM against heat-killed ΔXIII bacteria than sera from non immunized mice (control, treated with PBS) as determined by ELISA. No antibody to heat-killed ΔXIII bacteria was detected in sera from control mice (reciprocal titer, <1:20). Error bars represent standard deviation between individual antibody titers. Statistical analysis was carried out using a two-way analysis of variance combined with the Bonferroni test. *** $P < 0.001$. B) Opsonization with immune serum enhances the uptake of *Salmonella* by murine macrophage cells. Opsonization and phagocytosis of *S.* Typhimurium143/09 by J774.2 macrophages was tested by counting the number of phagocytosed bacteria that had been previously mixed with 10% immune serum obtained 28 days post immunization (IM), pre-immune serum (PreIM) or PBS. The relative bacterial uptake of bacteria mixed with serum with respect to the uptake of bacteria suspended in PBS (value of 1.0) is represented. Results from duplicates on three separate days are shown. Statistical analysis was performed using the Mann–Whitney test. *$P < 0.05$.

Since both humoral and cellular responses are necessary for immunity to oral challenge with virulent *S.* Typhimurium (Simon *et al.*, 2011), we next determined serum levels of IFN-γ (a prototype Th1 cytokine), as well as IL-10 and IL-5 (prototype Th2 cytokines). Remarkably, significant IFN-γ and IL-10 levels were detected in pooled sera from ΔXIII immunized animals and not from PBS treated mice (Fig. 5A). In particular, 14 days after immunization, IL-10 levels in sera from ΔXIII immunized mice were much higher than in sera from control mice ($p<0.001$). These levels decreased over time, although 28 days after immunization, they were still significantly higher than in control sera ($p<0.01$). As regards IFN-γ, it was also significantly present in sera from ΔXIII immunized mice ($p<0.05$) collected 14 and 28 days after immunization. On the other hand and in the case of IL-5, it is important to note that this cytokine was barely detectable in all samples analyzed.

To confirm the results obtained from sera and to evaluate the ΔXIII specific stimulation of cytokines production by spleen cells from immunized mice, we performed the following experiments. Supernatants of stimulated splenocytes with 10^7 cfu of heat-killed ΔXIII bacteria were analyzed for the production of IL-2 (prototype Th1 cytokines), IL-10 (prototype Th2 cytokines), and the Th17-associated cytokine IL-17. Remarkably, levels of the three cytokines were significantly higher in supernatants from splenocytes of immunized mice than in supernatants from splenocytes of control mice (Fig. 5B). It is important to note that differences were higher in the case of IL-2 and IL-17 production ($p<0.001$) than in the case of IL-10 ($p<0.05$). Also, an ELISPOT assay was carried out to investigate IFN-γ production and results showed that splenic lymphocytes from mice immunized with ΔXIII strain produced significantly more levels of IFN-γ secreting T cells than those of control mice ($p<0.001$) (Fig. 5C). Finally, we used flow cytometry coupled with stimulations with heat-killed ΔXIII bacteria

to measure IFN-γ and TNF-α production by CD4 and CD8 T cells (Fig. 5D and S1 Fig.). A strong CD4 T cell response specific of ΔXIII heat-killed bacteria was detected in AXIII immunized mice. Curiously, IFN-γ and TNF-α production was not detected in CD8 T cells (Fig. 5D, left). It is likely that the use of heat-killed bacteria for the intracellular detection of cytokines might have hindered the accurate analysis of the CD8 T cell response, since heat-killed bacteria are not efficiently processed and presented by MHC-I molecules (Lo *et al.*, 2004). The assessment of the CD8 T cell response induced by AXIII requires further work that involves the identification of bacterial antigens and/or the use of recombinant bacteria expressing reporter antigens.

Overall, these data indicate that mechanisms underlying ΔXIII mediated protection include the production of opsonizing antibodies and a mixed T cell response characterized by significant levels of IFN-γ, TNF-α, IL-2, IL-17 and IL-10.

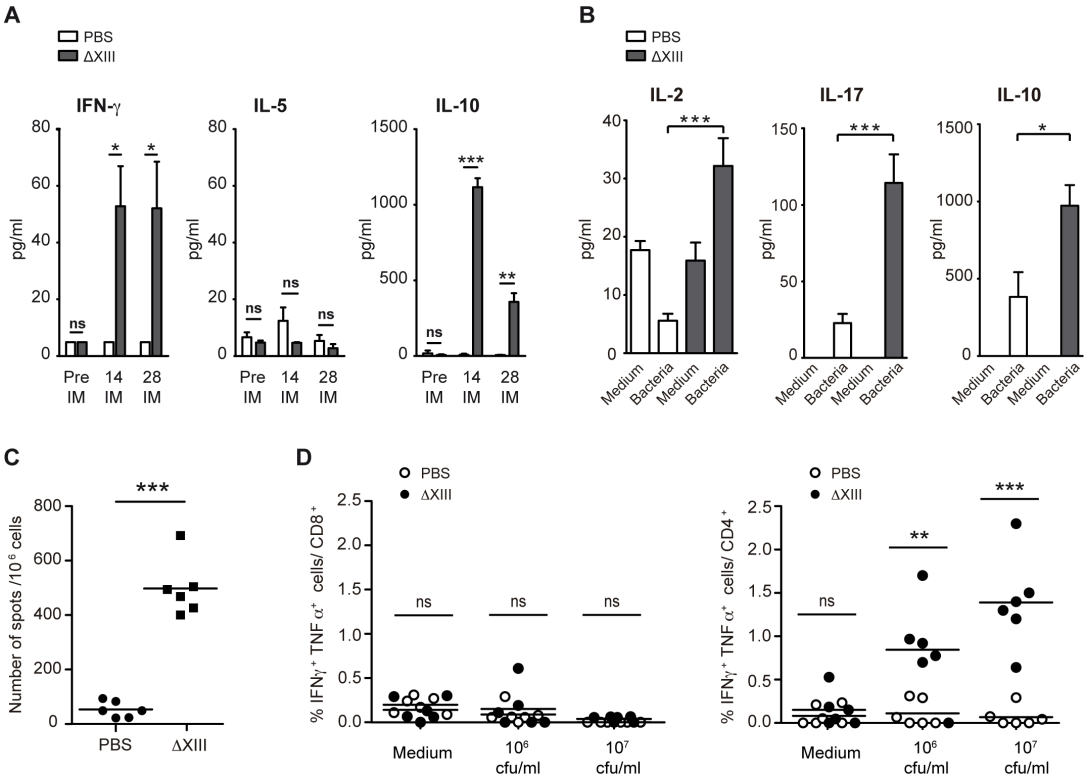

Fig. 5. ΔXIII induced cellular immune responses. A) Sera from ΔXIII immunized mice present significantly higher levels of interferon gamma (IFN-γ) and IL-10 than sera from the control group, treated with PBS. Quantification (pg/ml) of IFN-γ, IL-5 and IL-10 in pooled sera from the control group (PBS) and from the ΔXIII immunized group was carried out using respective ELISA kits. Error bars represent standard deviation between duplicate wells. Statistical analysis was carried out using a two-way analysis of variance combined with the Bonferroni test. ns=no significant difference; *P < 0.05; ** P < 0.01; *** P < 0.001. B) ΔXIII immunization induces production of cytokines in splenocytes. 28 days postimmunization, splenocytes were harvested from control and ΔXIII immunized mice and restimulated for 48 h with 10^7 cfu of heat-killed ΔXIII bacteria. RPMI media was used as a non-stimulating control. Cell supernatants were harvested and analyzed for IL-2, IL-10 and IL-17 production using respective ELISA kits. Statistical analysis was carried out using the unpaired Student *t* test. C) Numbers of IFN-γ producing cells in spleens from control and ΔXIII immunized mice were determined by ELISPOT assay. Splenocytes were harvested from all mice at 28 days after immunization. The results are expressed as spots per million splenocytes minus background from cells stimulated with 10^7 cfu of heat-killed ΔXIII bacteria. Each point represents an individual mouse. Lines indicate the mean of the replicates. Significant differences between groups are indicated and were determined using the unpaired Student *t* test. *** P < 0.001. D) CD4+ T cells produce IFN-γ and TNF-α upon ex vivo re-stimulation following vaccination with ΔXIII. 28 days after immunization, splenocytes from control and ΔXIII immunized mice were plated and stimulated for 6 hours with medium, 10^6 or 10^7 cfu of heat-killed ΔXIII bacteria. Cells were surface stained with anti-CD4 and anti-CD8 mAbs and intracellularly stained with anti-IFN-γ and anti-TNF-α antibodies. Shown are percentages of IFN-γ and TNF-α cells out of total CD4+ and CD8+ T cells. Each point represents an individual mouse. Lines indicate the mean of the replicates. Significant differences between control and vaccinated groups are indicated and were determined using the unpaired Student *t* test. ns=no significant difference; ** P < 0.01; *** P < 0.001.

Evaluation of ΔXIII as a live DIVA vaccine

A *Salmonella* ideal vaccine must not interfere with control programs, in the sense that infected animals with virulent field *Salmonella* strains must be differentiated from vaccinated animals. Therefore, we next aimed at exploring the properties of ΔXIII strain as a DIVA (Differentiation of Infected and Vaccinated Animals) vaccine. Since ΔXIII strain is a multiple mutant carrying deletions in *rpoS* and also in all GGDEF domain proteins encoding genes, we investigated the possibility of using the absence of one GGDEF protein as a DIVA marker. Interestingly, the GGDEF domain protein encoding gene *sen4316* does not show any orthologous gene in other enterobacteriaceae and thus, we found conceivable the idea of using the antibodies raised against this protein, that could only be developed in animals that had been colonized by a natural wild type strain but would be absent in vaccinated ones, in order to differentiate infected and vaccinated individuals. To prove so, groups of 10 mice were orally inoculated with a single dose of 10^7 cfu of ΔXIII or a sublethal dose (10^4 cfu) of the wild type strain and sera samples were obtained periodically up to day 44 post inoculation. Concurrently, we designed a specific ELISA assay in which a 6-His–tagged recombinant version of SEN4316 was used as the bound antigen. This assay showed that titers of antibodies against SEN4316 in pooled sera from animals infected with the wild type strain rose gradually after infection. On the contrary, and as expected, titers in pooled sera proceeding from ΔXIII immunized animals remained negative (Fig. 6A).

In addition to the immunoassay, we also explored the possibility of using the *sen4316* gene as a positive genetic marker to identify vaccinated animals through PCR. To do so, faecal samples from each group were pooled and collected periodically and bacterial genetic material was extracted. Oligonucleotide DIVA-1 was designed so that the 5´end of the oligonucleotide hybridizes to a DNA sequence downstream *sen4316* whilst

the 3´end of the oligonucleotide hybridizes to a DNA sequence upstream *sen4316* (Fig. 6B). Oligonucleotide DIVA-2 hybridizes to a DNA sequence downstream *sen4317* (Fig. 6B). PCR conditions were designed in such a way that amplification only took place if the ΔXIII genome acted as template DNA. On the other hand, amplification of the *Salmonella* specific gene *invA* with oligonucleotides invA-fw and invA-rv was used as an internal positive control in all samples. Faecal samples proceeding from animals infected with the wild type strain resulted *invA* positive, DIVA negative, while samples proceeding from vaccinated animals resulted *invA* positive, DIVA positive (Fig. 6B).

These results confirmed that ΔXIII can be considered a DIVA vaccine since the GGDEF protein SEN4316 enables discrimination of vaccinated animals either by immunological or by molecular non sophisticated approaches.

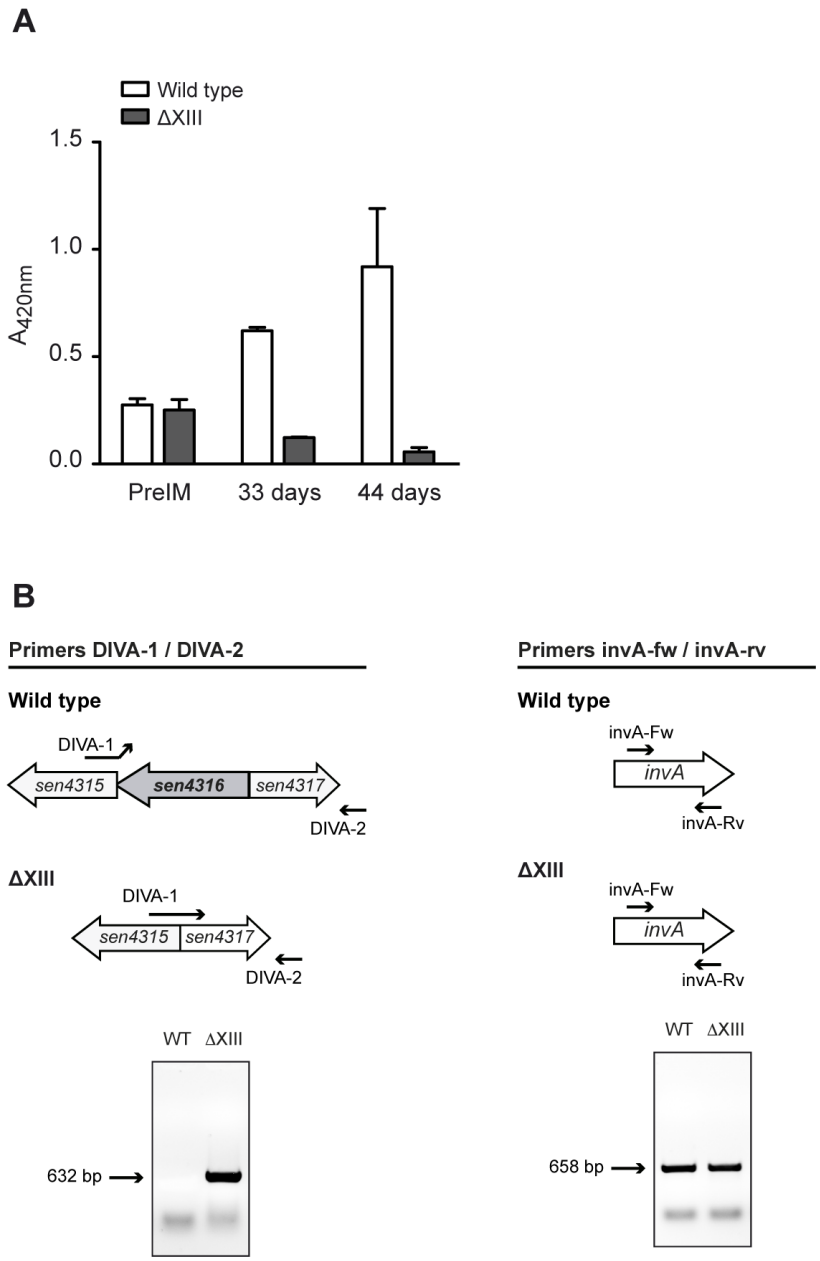

Fig. 6. ΔXIII is a DIVA vaccine that allows differentiation of infected and vaccinated animals. (A) SEN4316 based ELISA of pooled sera from wild type and ΔXIII infected animals, obtained before infection (PreIM) and 33 and 44 days post infection. Sera from animals infected with the wild type strain and not with ΔXIII present antibodies against the SEN4316 protein. Error bars represent standard deviation between triplicate wells. (B) DNA from pooled faecal samples from wild type and ΔXIII infected animals, collected at day 1, 7, 14 and 21 post infection, were analyzed by PCR. Amplification of stool DNA with primers DIVA-1 and DIVA-2 allowed the identification of vaccinated animals with ΔXIII strain. Amplification with primers invA-fw and invA-rv served as a control of the presence of *Salmonella* DNA. Results shown are representative of results obtained throughout time, since *Salmonella* DNA was present in faecal samples since day one post infection.

Survival of ΔXIII in the environment

One important drawback of live attenuated vaccines is that the strain, once excreted by vaccinated animals, may persist for long periods in the environment. To determine whether this might be a handicap to the use of ΔXIII strain, phenotypic traits involved in environmental persistence, such as resistance to desiccation in the absence of nutrients and biofilm formation were assessed.

Results showed that almost 100% of wild type bacteria remained viable after 12 days under waterless conditions. On the contrary, both Δ*rpoS* and ΔXIII strains showed to be dramatically sensitive to desiccation, since, at the end of the experiment, there were not any live bacteria left (Fig 7A). Biofilm formation capacity was analyzed by incubating bacteria in LB broth under static and room temperature conditions for 72 hours and also, by analyzing colony morphology on plates containing the Congo Red dye (Fig. 7B). Δ*rpoS* and ΔXIII strains did not form a biofilm in LB and showed a "saw" (smooth and white) morphotype, which corresponds with the inability to produce cellulose and fimbriae, the main components of the *Salmonella* biofilm matrix, and thus a total absence of multicellular behavior.

These results demonstrate that ΔXIII might be easily eliminated from the environment when excretion by vaccinated animals occurs.

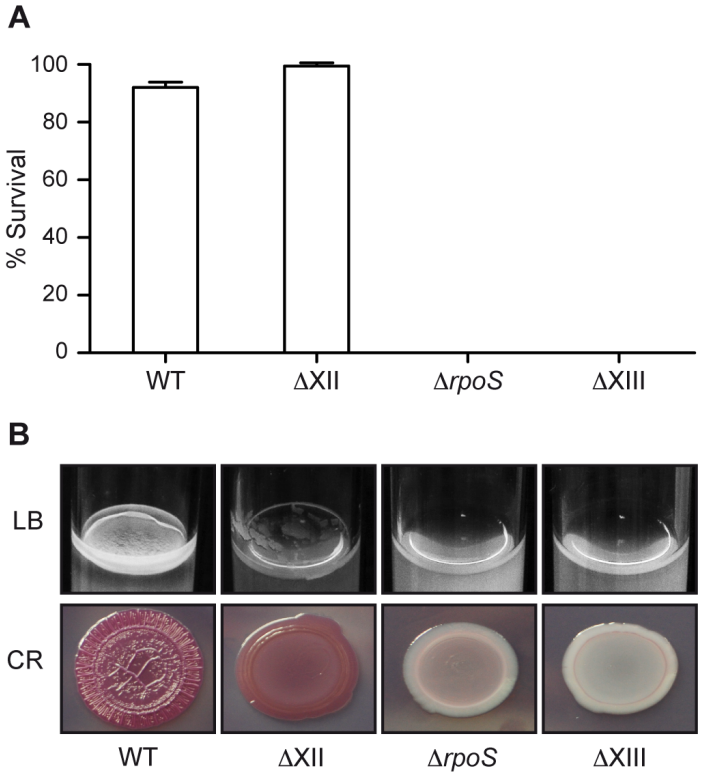

Fig 7. ΔXIII strain is unable to form a biofilm and does not survive after twelve days of desiccation in the absence of nutrients. (A) Survival of the wild type strain, new ΔXII (absence of c-di-GMP), the single Δ*rpoS* mutant and the vaccine candidate ΔXIII after twelve days of desiccation. Surviving bacteria were enumerated by viable plate counts, and their numbers were compared with those of initial inocula, which defined 100% survival. Means and standard deviations of results from three independent experiments are shown. (B) Biofilm formation capacity of the same strains. Biofilm phenotypes were visualized after growth in LB medium conditions and on congo red agar plates.

DISCUSSION

C-di-GMP is recognized as a universal bacterial second messenger implicated in the regulation of a large number of cellular functions, including cell cycle progress, differentiation, biofilm formation and dispersion, motility and virulence. As regards virulence, it is generally assumed that high levels of c-di-GMP in bacterial cells promote biofilm formation and the establishment of chronic infections, whilst low c-di-GMP levels promote virulence factor synthesis and thus, acute infections (Tamayo *et al.*, 2007; Povolotsky and Hengge, 2012; Römling *et al.*, 2013). In contrast to this general view, our results with new ΔXII strain, which lacks all GGDEF domain proteins and thus cannot make c-di-GMP, have demonstrated that, in *Salmonella*, absence of c-di-GMP signaling is detrimental for the development of an acute infection. Virulence decrease shown by ΔXII strain was subtle but consistent enough to indicate that either an specific GGDEF domain protein or a certain c-di-GMP related phenotype or c-di-GMP itself might be required at some stage of the *Salmonella* acute infection. These results are in line with recent evidences that are revealing a more complicated picture about the relationship of the c-di-GMP network and virulence than expected. Individual mutants in not only c-di-GMP degrading enzymes (phosphodiesterases; PDEs) but also synthesizing enzymes (diguanylate cyclases; DGCs) have shown to display attenuation in infection models (Kulasakara *et al.*, 2006; Ahmad *et al.*, 2011; Allombert *et al.*, 2014), suggesting that opposite c-di-GMP-metabolizing processes might play a role at different disease stages (Römling *et al.*, 2013). Also, putative DGCs and PDEs with no c-di-GMP metabolizing activity have been described to affect virulence (Shang *et al.*, 2009; Stewart and Cummings, 2011; Ahmad *et al.*, 2013; Spurbeck *et al.*, 2013; Yang *et al.*, 2014). Accordingly, virulence defect of ΔXII strain might be explained by the fact that several *Salmonella* GGDEF domain proteins harbor a degenerated consensus signature and

might somehow control virulence independently of c-di-GMP levels. With respect to the participation in virulence of a particular c-di-GMP controlled phenotype, we showed in a previous study that production of cellulose is not involved in the virulence of *S.* Enteritidis in BALB/c mice infected orally or intraperitoneally (Solano *et al.*, 2002). However, a very recent report by Pontes et al. (Pontes *et al.*, 2015) has demonstrated that *Salmonella* makes cellulose inside macrophages and that its synthesis hinders *Salmonella* replication inside host cells. Thus, a cellulose mutant strain was hypervirulent in C3H/HeN mice injected intraperitoneally. In any case, results of both studies do not correspond with results obtained here which show that ΔXII strain, that cannot synthesize cellulose, is less virulent than the wild type strain. Hence, virulence defect of ΔXII strain cannot be attributed to a lack of cellulose synthesis. With regard to the direct contribution of c-di-GMP to virulence, it is known that it binds to the eukaryotic proteins STING and DDX41 (Woodward *et al.*, 2010; Burdette *et al.*, 2011; Parvatiyar *et al.*, 2012; Valle *et al.*, 2013), leading to induction of type I interferons and thus, apparently contributing to successful pathogen elimination. This fact, again, does not agree with ΔXII strain being less virulent than the wild type strain. On the contrary, ΔXII virulence attenuation might be related to a very recent research of Li et al. (Li *et al.*, 2015) finding that c-di-GMP directly binds to human siderocalin (LCN2). LCN2 sequesters bacterial siderophores to prevent iron acquisition and inhibit bacterial growth under iron-limited conditions (Goetz *et al.*, 2002; Flo *et al.*, 2004). Li et al. showed that c-di-GMP competes with bacterial siderophores to bind to LCN2, therefore, preventing LCN2 from inhibiting bacterial growth. These results indicated that c-di-GMP interferes with LCN2 to ensure bacterial survival during infection and that inhibition of c-di-GMP production should attenuate virulence. Further detailed analysis of the contribution of the c-di-GMP signaling network to *Salmonella* virulence will be required to figure out

the reasons why ΔXII strain presents an attenuated phenotype.

Once we cleared up the effect of the absence of c-di-GMP signaling in *Salmonella* virulence, and with the final aim of constructing a novel vaccine candidate, we performed a mutation of the *rpoS* gene in ΔXII, giving rise to strain ΔXIII. Absence of all GGDEF domain proteins and also of RpoS had a synergistic attenuating effect that made ΔXIII extremely safe and capable to protect vaccinated mice from a lethal dose challenge of a wild type *S.* Typhimurium strain. The cross-talk between T and B cells is of fundamental importance for the establishment of solid acquired immunity to salmonellosis (Mastroeni and Ménager, 2003). Accordingly, mice protection with ΔXIII strain was found to occur via different immune responses including *Salmonella*-specific IgGs that showed a moderate opsonic activity and high levels of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-2, IL-17 and also of the anti-inflammatory cytokine IL-10. IFN-γ has a crucial role for clearing infections caused by intracellular bacteria, given that its major effect is macrophage recruiting and that activation and high titters of this cytokine generally implies a strong immune response that is accompanied by T-lymphocyte expansion (Mastroeni *et al.*, 2000; Dougan *et al.*, 2011). Thus, mice treated with anti- IFN-γ antibodies and also, mice and humans genetically deficient in immunity mediated by IFN-γ are highly susceptible to *Salmonella* (Muotiala and Mäkelä, 1990; VanCott *et al.*, 1998; Jouanguy *et al.*, 1999). With respect to IL-17, it is emerging as a central element of mucosal immunity to microbial challenge since it enhances basic innate barrier defenses such as antimicrobial peptide production and neutrophil recruitment (Rubino *et al.*, 2012). Accordingly, *S.* Typhimurium-infected IL-17-receptor-A-deficient mice have lower amounts of inflammatory cytokines, less neutrophil recruitment and increased bacterial translocation to the spleen and mesenteric lymph nodes (Raffatellu et al., 2008). On the other

hand, IL-10 is a well-characterized anti-inflammatory cytokine that inhibits synthesis of Th1 cytokines and proliferation of T cells, preventing the detrimental consequences of systemic inflammation (Moore et al., 2001). The presence of IL-10 in ΔXIII immunized animals suggests that the immune response was tightly balanced (Schulte et al., 2011; Voinnet, 2011) and that ΔXIII might exploit the immunosupresive effects of IL-10 to persist in mice organs, eliciting a long-lasting immunity (Cyktor and Turner, 2011).

Up to date there is no ideal vaccine available for control of salmonellosis in farm animals. Such a vaccine should be cheap, minimally reactive and preferably live and invasive but still safe to induce strong immunity (Singh, 2009). Furthermore, the use of attenuated vaccines as veterinary drugs can only be recommended if differentiation of vaccinated from infected animals (DIVA) is feasible in a basic bacteriology laboratory (Selke et al., 2007; Singh, 2009). In response to this need, antigenic, genetic and phenotypic markers have been incorporated into or eliminated from attenuated vaccines (Henderson, 2005; Leyman et al., 2011). However, bacterial “tagging” is normally achieved through genetic engineering methods and serious concerns are being raised about the effects that genetically modified microbes (GMMs) may have if massively used or released into the environment (Moe-Behrens et al., 2013). In order to avoid the use of antibiotic markers, ΔXIII strain is the result of thirteen consecutive deletion steps in which an allelic exchange strategy with sacB as the counterselectable marker was used (Solano et al., 2009; Zorraquino et al., 2013). Thus, ΔXIII does not contain any trace of exogenous DNA and is indistinguishable from a naturally occurring mutant, fact that eludes its classification as a GM organism according to the European Directive [EC] 2001/18. Amongst the thirteen candidates to become a negative selectable marker, we found several reasons for choosing the GGDEF protein SEN4316. In contrast to other GGDEF proteins, SEN4316 is conserved in

all *S. enterica* serovars and thus, it can be considered a “broad spectrum” marker since seroconversion is likely to occur upon infection of animals with any *Salmonella* serovar, thereby activating the DIVA function (Selke *et al.*, 2007). On the other hand, *E. coli* and other gram negative species lack this member of the GGDEF family (data not shown). We designed a SEN4316 based ELISA that allowed the serological discrimination of vaccinated from infected mice. In addition, and since such DIVA strategy identifies infected animals, we developed a very simple molecular method to positively identify vaccinated animals. However, its application would be restricted to the period of time in which the vaccine is present in faeces.

Because live vaccines can be potentially excreted into the environment by immunized animals, another condition of increasing interest in vaccine development is the easiness to eliminate it from the environment (Leyman *et al.*, 2012; Desin *et al.*, 2013). Undoubtedly, this is one of the strengths when considering ΔXIII strain as a live vaccine candidate since it is totally incapable of synthesizing a protective biofilm matrix and lacks RpoS, a regulator that is indispensable to mount an adequate response against environmental stresses. Accordingly, less than two weeks were sufficient to end up with 100% of ΔXIII bacteria under waterless conditions.

Salmonella mutants of reduced virulence that still display high immunogenicity have already been described (Denagamage *et al.*, 2007; Karasova *et al.*, 2009; Arguello *et al.*, 2012; Desin *et al.*, 2013). We propose that the c-di-GMP signaling and RpoS defective *Salmonella* strain described here, which shows a delicate safety-immunogenicity balance, promising DIVA features and can be easily eliminated from the environment is a noticeable new candidate to include in this list. Future work will be conducted to analyze its potential use in field vaccination trials.

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SUPPLEMENTARY MATERIAL

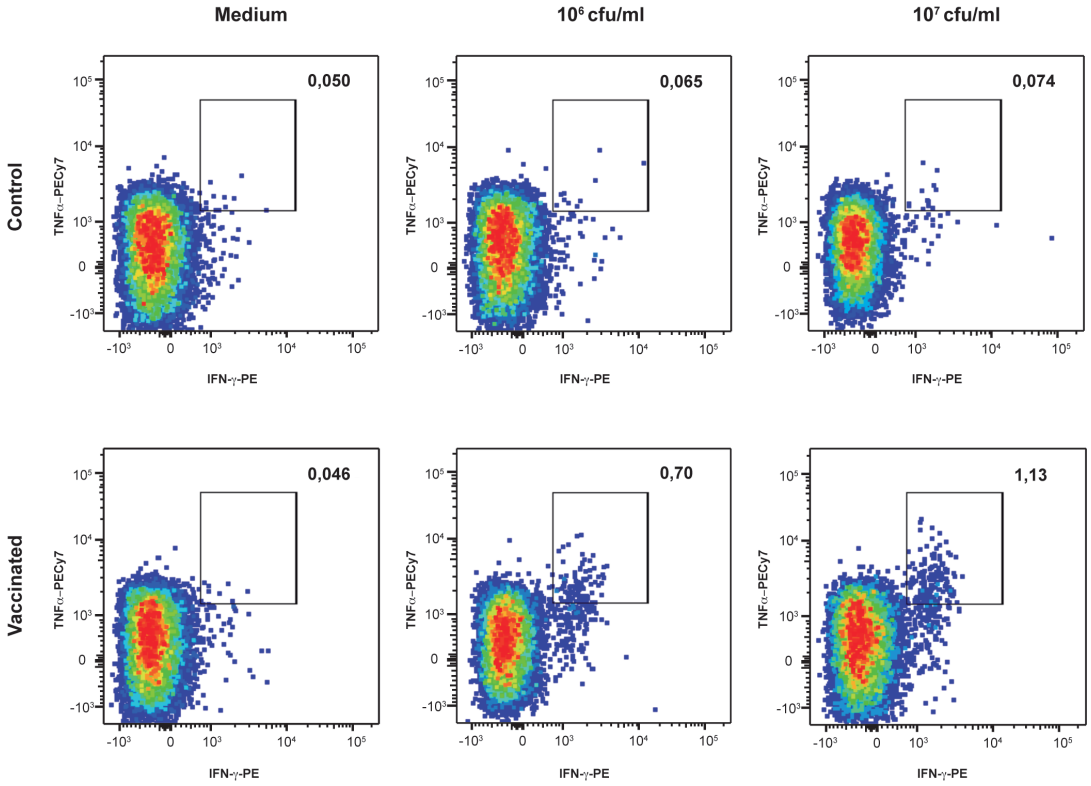

Fig. S1. Representative IFN-γ and TNF-α/CD4 staining for ΔXIII stimulated splenocytes. 28 days after immunization, splenocytes from control and ΔXIII immunized mice were plated and stimulated for 6 hours with medium, 10^6 or 10^7 cfu of heat-killed ΔXIII bacteria. Cells were surface stained with anti-CD4-FITC antibody and intracellularly stained with anti IFN-γ-PE and anti-TNF-α-PE-Cy7 mAbs. Shown are percentages of IFN-γ and TNF-α cells out of total CD4+ T cells.

CHAPTER II

Evaluation of the role of c-di-GMP and STING sensing pathway during *Salmonella* infection

SUMMARY

The endoplasmic protein stimulator of IFN genes (STING) has been recognized as an eukaryotic cyclic diguanylate monophosphate (c-di-GMP) receptor. Upon c-di-GMP sensing, STING activates a signaling cascade to stimulate a type I interferon response. Therefore, synthetic c-di-GMP has been shown to be a potent stimulator of innate immunity in eukaryotic organisms and thus, there has been an ongoing focus on using c-di-GMP as an adjuvant to improve vaccine efficiency. However, whether the c-di-GMP/STING pathway functions in host defense mechanisms against pathogens with the ability to synthesize c-di-GMP is an issue that remains unclear. *Salmonella* is a facultative intracellular pathogen that produces c-di-GMP to control critical cellular processes such as biofilm formation, motility and virulence. A *Salmonella* strain unable to synthesize c-di-GMP, named ΔXII is biofilm-deficient and shows a moderate attenuation in virulence. Here, through the use of *STING*$^{-/-}$ bone marrow-derived macrophages and *STING*$^{-/-}$ mice we report that, *Salmonella* stimulates the IFN response via STING in murine macrophages independently of c-di-GMP. On the other hand, STING deficient mice were equally susceptible than wild type mice to a *Salmonella* infection in a systemic murine infection model. Altogether, our results indicate that the c-di-GMP/STING pathway does not play a significant role in host defense against a *Salmonella* infection.

INTRODUCTION

Innate immunity is the first line of defense against infection. It recognizes and provides nonspecific defense against pathogens. The recognition depends on receptors (pattern-recognition receptors, PRRs) that bind molecules expressed by microbial organisms that are chemically different from those produced by host cells (pathogen-associated molecular patterns, PAMPs) (Iwasaki and Medzhitov, 2010). Thus, binding of the PAMP to the PRR triggers signaling pathways not only to alert the immune system to the presence of infection but also to initiate adaptive immune responses through activation of dendritic cells and antigen presentation (Medzhitov and Janeway, 2000). Recent studies have shown that bacterial cyclic dinucleotides (c-di-NMPs) act as PAMPs eliciting a host type I interferon (IFN) innate immune response characterized by activation of IFN production (McWhirter *et al.*, 2009; Woodward *et al.*, 2010; Abdullah *et al.*, 2012; Burdette and Vance, 2013). Such bacterial c-di-NMPs include cyclic diguanylate monophosphate (c-di-GMP), cyclic diadenylate monophosphate (c-di-AMP) and 3´3´ cyclic-GMP-AMP (cGAMP) (Woodward *et al.*, 2010; Davies *et al.*, 2012; Burdette and Vance, 2013). Two distinct PRRs able to directly bind bacterial c-di-NMPs and mediate the induction of type I IFN have been described. Binding of c-di-NMPs to a dimer of the stimulator of interferon genes (STING) results in the activation of the IRF3 transcription factor, which is required for host transcriptional activation of type I IFNs (Sauer *et al.*, 2011; Jin *et al.*, 2011; Burdette *et al.*, 2011). More recently, DDX41, a DEAD-box helicase, has been shown to directly bind c-di-NMPs and mediate the induction of IFN through a pathway that likely converges upstream of STING (Parvatiyar *et al.*, 2012; Bowie, 2012). Remarkably, STING dependent type I IFN production can also be strongly induced by an eukaryotic endogenous cyclic nucleotide. A very recently identified cytosolic DNA sensor, cGAS (cyclic-GMP-AMP synthase), is activated upon binding

to DNA and as a consequence, produces an unusual cyclic dinucleotide, 2´3´ cyclic-GMP-AMP (cGAMP), from ATP and GTP substrates. The cGAMP product then acts as a second messenger by binding and activating STING (Sun *et al.*, 2013; Wu *et al.*, 2013; Shi *et al.*, 2015) (Fig. 1).

Studies for evaluating the immunostimulatory and immunomodulatory properties of c-di-AMP and c-di-GMP confirm that these molecules exhibit potent adjuvant properties (Hu *et al.*, 2009; Chen *et al.*, 2010; Libanova *et al.*, 2011; Blaauboer *et al.*, 2014). With regard to c-di-GMP, its direct administration as a pure molecule has been shown to induce innate immune responses that can enhance protection of mice against challenges with *Klebsiella pneumonia* (Karaolis *et al.*, 2007), *Staphylococcus aureus* (Brouillette *et al.*, 2005; Hu *et al.*, 2009), *Bordetella pertussis* (Elahi *et al.*, 2014), *Acinetobacter baumannii* (Zhao *et al.*, 2011) and *Streptococcus pneumoniae* (Yan *et al.*, 2009). Also, bacterial diguanylate cyclases have been incorporated into adenovirus vaccines in order to foster production of c-di-GMP as well as proinflammatory responses in mice (Koestler *et al.*, 2014; Alyaqoub *et al.*, 2016).

In spite of these already known immunostimulatory properties of c-di-NMPs as adjuvants, there is little information about these dinucleotides being actually produced *in vivo*, during a bacterial infection process, and also about their exact role in pathogenicity. On one hand, c-di-NMPs would need to be released from bacteria at sufficient levels to activate host receptors. In the case of c-di-AMP, it has been shown that *Listeria monocytogenes* releases this nucleotide to the cytoplasm through two major multidrug resistance (MDR) efflux pumps (Woodward *et al.*, 2010). However, with respect to c-di-GMP, there is no evidence suggesting the secretion of this nucleotide outside bacteria. On the other hand, there is currently not any developed method to measure the accumulation of c-di-NMPs in infected tissues and thus, it is difficult to provide evidence of c-di-

NMPs production during *in vivo* bacterial infections. One remarkable step forward in the analysis of c-di-NMPs role during infection and their interaction with the immune system has been the generation of STING mutant mice (*Goldenticket* or *Gt* mice) (Sauer *et al.*, 2011). This mouse strain harbors a point mutation (T596A) in STING that results in an isoleucine-to-asparagine substitution (I199N) in the STING protein, rendering a nonfunctional allele that fails to produce detectable protein. *Gt* bone marrow-derived macrophages do not make IFN-β in response to either purified c-di-GMP and c-di-AMP or to *Listeria monocytogenes* infection (Sauer *et al.*, 2011; Woodward *et al.*, 2010). *Gt* mouse lung fibroblasts fail to induce a type I IFN response to *Chlamydia trachomatis*, which also synthesizes c-di-AMP (Barker *et al.*, 2013). However, although *Gt* mice present a significantly lower amount of IFN-β in the serum during a *L. monocytogenes* infection, they present a bacterial burden in spleens similar to wild type mice and thus, are not significantly more resistant to this bacterial infection (Sauer *et al.*, 2011).

In summary, there is convincing support for the idea that c-di-NMPs are detected by the innate immune system as a novel PAMP type. However, a deeper insight into the mechanisms of c-di-NMPs detection by the host cell and the role that bacterial c-di-NMPs play during infection is needed. In the previous chapter, we described that a *Salmonella* strain unable to synthesize c-di-GMP, the ΔXII strain, is slightly less virulent than the wild type strain. Therefore, we decided to investigate whether this attenuation had a connection with the STING recognition pathway, and the role, if any, of the STING signaling pathway during *Salmonella* infection, making use of *Gt* bone marrow-derived macrophages and *Gt* mice.

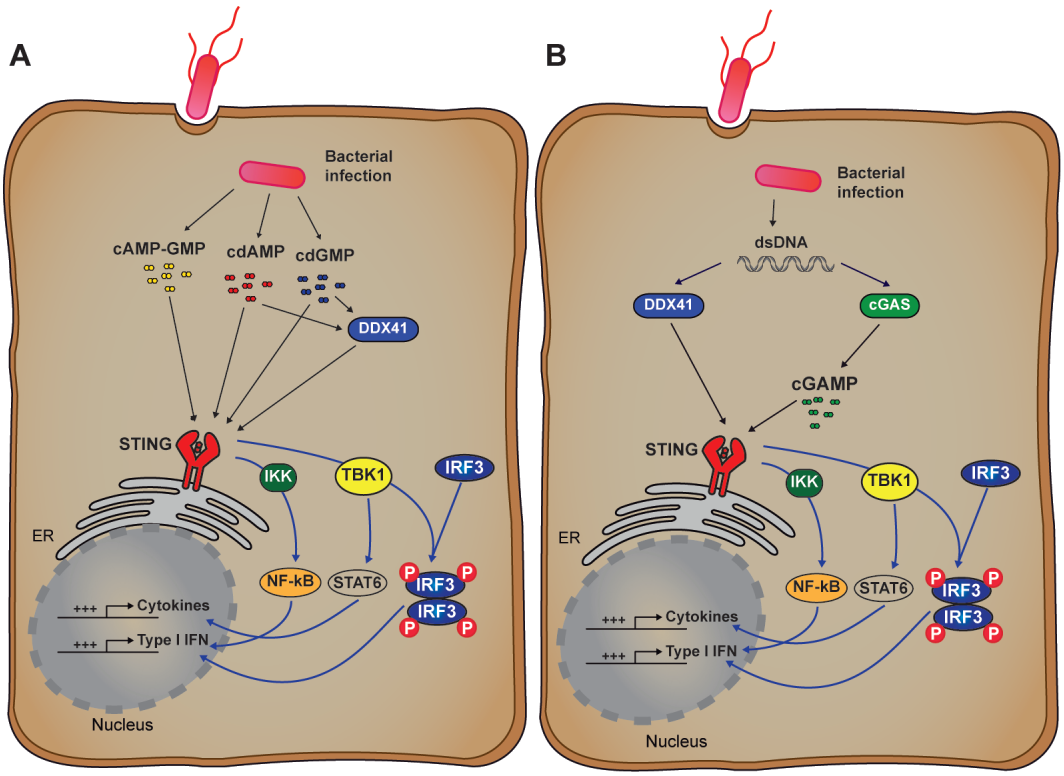

Fig. 1: Stimulation of type I IFN production through DDX41 helicase, cGAS and STING activation by c-di-NMPs and DNA during bacterial infections. Bacterial infections of eukaryotic cells lead to accumulation of cyclic dinucleotides and/or extracellular DNA that signal the presence of pathogens to the cellular immune system. A) Bacterial cyclic dinucleotides, that include c-di-AMP (cdAMP), c-di-GMP (cdGMP), and 3´-5´,3´-5´cGAMP (cAMP-GMP) interact with DDX41 helicase and STING. B) Cytosolic dsDNA is recognized by DDX41 and cGAS inducing the production of 2´-5´, 3´-5´cGAMP to stimulate STING. Binding of STING to an activating ligand induces conformational changes in STING and the following STING relocation to discrete foci in the cell cytosol. Activated STING recruits TBK1 and IKK kinases, which in turn activate IRF-3, STAT6, and NF-kB. Upon translocation of activated transcriptional factors to the nucleus, they bind to their corresponding promoters, resulting in induction of type I IFN and cytokines.

MATERIAL AND METHODS

Bacterial strains and culture media

The wild type strain S. *enterica* subsp. *enterica* serovar Enteritidis 3934 (*S.* Enteritidis 3934) and its derivative ΔXII strain were grown in LB broth. The strains and oligonucleotides used in this chapter are described in Table 1. The media used for bone marrow macrophages differentiation is detailed below:

Bone Marrow Macrophage Differentiation Media (BMDM)

Iscove´s modified MEM (Invitrogen)	500 ml
DFBS (Gibco)	50 ml
L-cell conditionated media	150 ml
100x Non-essential amino acids (Gibco)	5 ml
50 mM b-mercaptoethanol(Gibco)	0,45 ml
100x Penicillin/Streptomicin (Gibco)	5 ml

L-cell Conditioned Media

2,5 x10^5 L-cells were seeded on 75 cm^2 flask containing 50 ml of IMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin. When cells reached confluency (6-7 days), media was harvested, filtered and stored at 4ºC until use.

Extraction and culture of Bone Marrow Derived Macrophages (BMDM)

Bone marrow-derived macrophages (BMDM) were prepared form femurs of C57BL/6J wild type and *STING*$^{-/-}$ mice. Femurs and tibias were surgically removed, freed from muscles and tendons, and briefly suspended in PBS. Ends were cut, the marrow was flushed with PBS and the cell suspension was filtered over a 70-µm cell strainer (BD Falcon) and centrifuged for 5 min at 1800 rpm. Red blood cells were removed using ACK

(Ammonium-Chloride-Potassium) lysing buffer. After two washes, cells were resuspended at a concentration of $1x10^6$ ml^{-1} in Bone Marrow Macrophage Differentiation Media, seeded in tissue treated plates and incubated at 37ºC with 5% CO_2. Two days after, media was changed and cells were incubated again during two days. On day 5, adherent cells were harvested, centrifuged, washed, and resuspended in Roswell Park Memorial Institute 1640 medium (RPMI) (Gibco) supplemented with 10% FBS (Invitrogen) and 1% Penicillin/Streptomycin (Gibco). After counting the cells, macrophages were seeded at a density of 5 x 10^5 cells per well in 12-well plates, 24 h prior to infection.

Macrophages infection: bacterial uptake and survival

Bone marrow derived macrophages (BMDMs) from C57BL/6J wild type and *STING*$^{-/-}$ mice were propagated in (RPMI) (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin/Streptomycin (Gibco). Macrophages were seeded at a density of 5 x 10^5 cells per well in 12-well plates 24 h prior to infection. *Salmonella* overnight cultures grown at 37ºC in LB broth were sonicated (30 sec; potency 3; Branson sonifier 250; microtip) and diluted 1:100 in LB broth. Cultures were incubated at 37ºC in an orbital shaker (200 r.p.m) to an OD_{600nm} of 1 and the suspension was sonicated again and washed twice with RPMI deprived of serum. Macrophages were then infected with *Salmonella* strains at a multiplicity of infection of approximately 10:1 and plates were centrifuged at 1000 r.p.m. for 10 minutes at room temperature. After 20 min of phagocytosis, monolayers were washed twice with PBS and treated with gentamicin (100 µg ml^{-1}) for 2 h. To estimate phagocytosed bacteria, samples were then washed three times with sterile PBS and macrophages were lyzed with 1% (vol/vol) Triton X-100-PBS to release intracellular bacteria that were counted by plating 25 µl of serial dilutions onto LB plates. To assess bacterial

survival, medium was replaced by RMPI supplemented with 10% FBS and 12 μg ml^{-1} gentamycin and the cells were incubated at 37ºC. After 2 h, wells were washed twice with PBS and treated with Triton X-100 as indicated above. Experiments were done in triplicate on three independent occasions.

Macrophages immune response: determination of cytokines

BMDMs were infected as described above. Two and four hours after bacterial uptake, cells were collected for RNA extraction and cytokines levels were determined by qRT-PCR. Total cellular RNA was isolated using RiboPure™ RNA Purification Kit (Ambion™) according to the manufacturer's instructions. One microgram of each RNA sample was subjected per duplicate to 0,3 U/μl of DNAse TURBO DNA-Free™ (Ambion) treatment for 30 min at 37ºC. The enzyme was inactivated at 65ºC in the presence of EDTA 0.25 mM during 10 min. The RNA samples were reverse transcribed in the presence of the enzyme M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Applied Biosystems) according to the manufacturer's instructions.

qRT-PCR was performed in 96-well microplates using GoTaq® qPCR Master Mix (Promega) and 1μg of cDNA. Amplification and detection of the PCR products were performed using a C1000 Thermal Cycler (BioRad) with an initial denaturation at 95º C for 10 min and 40 cycles at 95º C for 15 s, 60ºC for 1 min and 72ºC for 25 s. Each sample was subjected to qRT-PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. The Ct values of the genes of interest were normalized (ΔCt) to an average Ct value of the house-keeping gene (GAPDH) and the relative expression of the gene of interest was calculated as $2^{-\Delta Ct}$. These expression levels were used for data analysis. Primers used in qRT-PCR are listed in Table 1.

Mice

C57BL/6J-Tem173gt/J heterozygous transgenic mice (named as Goldenticket) were purchased from Jackson Laboratory and supplied from the Gene Therapy lab of the Center for Applied Medical Research (CIMA). These Tem173gt/J chemically induced mutant mice carry a missense mutation in exon 6 of the transmembrane protein 173 (TMEM173 or STING) gene, which results in an isoleucine to asparagine change in aminoacid 199 (I-199-N), in the C-terminal of the protein, that functions as a null allele and fails to produce detectable protein (Sauer *et al.*, 2011). The heterozygous mice were interbred to obtain wild type and golden ticket homozygous litter-mates. Genotypes of mice were determined from ear tissue by genomic PCR with STING Fw and STING Rv oligonucleotides using a KAPA Mouse Genotyping Kit (KAPA Byosistems). DNA sequencing allowed differentiation of wild type (T596) and golden ticket mice (A596). Mice were bred and maintained under pathogen-free conditions in the animal facility of CIMA. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra (Ethical protocol 130-12).

Ileal loop infection assay

To compare the *in vivo* interaction of *Salmonella* strains with murine intestinal epithelial cells of wild type and *STING*$^{-/-}$ mice, the ligated ileal loop infection model was used as described previously, (Solano *et al.*, 2002) (Latasa *et al.*, 2005). Strains were incubated overnight under shaking conditions at 37ºC, suspended in PBS and sonicated (30 sec; potency 3; Branson sonifier 250; microtip) prior to infection. Each ileal loop was infected with only one strain to avoid possible interferences of strains with the host immune response during a coinfection experiment.

Colonization experiments

Colonization experiments were carried out with 8-week-old female C57BL6/J and C57BL6/J *STING*$^{-/-}$ mice in the animal facility of CIMA. In the case of intragastric inoculation, food and water were removed, twelve and two hours respectively, before the administration of bacterial suspension. Mice were prefed with 20 µl of 10% sodium bicarbonate 30 min before bacterial inoculation. Water and food were again supplied right after inoculation.

Strains were incubated overnight under shaking conditions at 37ºC, suspended in PBS and sonicated (30 sec; potency 3; Branson sonifier 250; microtip) prior to infection. Mice were inoculated intraperitoneally (i.p) or intragastrically (i.g) with 100 µl of bacterial suspensions containing 1 x 10^4 cfu or 1 x 10^8 cfu, respectively. The cfu of each strain in the inoculum (input) were quantified by plating dilution series on LB. Mice were euthanized after three days (i.p) or five days (i.g), and then dilution series of liver, spleen and gallbladder lysates were plated on LB agar for enumeration of cfu.

Statistical analyses

All statistical analyses were performed in GraphPad Prism 5.01. using a two-way analysis of variance combined with the Bonferroni test.

Table 1. Strains and oligonucleotides used in this study

Strain	Relevant characteristics	MIC[a]	Reference or source
***S*. Enteritidis** 3934	Wild-type clinical isolate	54	(Solano *et al.*, 1998)
***S*. Enteritidis** ΔXII	3934 Δ*adrA* Δ*sen1023* Δ*yeaJ* Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* Δ*yfeA* Δ*sen4316*	1324	(Zorraquino *et al.*, 2013)

Oligonucleotides for qPCR analysis of INF-β and TFN-α

Primer	Sequence 5´→3´
IFN-β Fw	ATGAGTGGTGGTTGCAGGC
IFN-β Rv	ACCTTTCAAATGCAGTAGATTCA
TNF-α Fw	CTTCCAGAACTCCAGGCGGT
TNF-α Rv	GGTTTGCTACGACGACGTGGG
GADPH Fw	TGCACCACCAACTGCTTA
GADPH Rv	GGATGCAGGGATGATGTTC
STING Fw	GCTCCAGGCCCGGATCCGAATG
STING Rv	CTGTTGGAATAAACCCGATTC

[a]Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra.

RESULTS

Salmonella DNA recognition by STING induces IFN-β production in macrophages independently of c-di-GMP.

Many intracellular pathogens induce expression of type I interferons through recognition of bacterial PAMPs by PRRs located at the cell surface and in the cytosol (Trinchieri, 2010). C-di-NMPs either produced by invading bacterial pathogens (such as c-di-AMP and c-di-GMP) or produced endogenously in response to foreign DNA (cGAMP) trigger type I IFN induction through direct interaction with the endoplasmic reticulum membrane protein STING (McWhirter *et al.*, 2009; Woodward *et al.,* 2010; Burdette *et al.*, 2011; Sun *et al.*, 2013).

To test the role of STING during a *Salmonella* infection and to evaluate the involvement of the lack of c-di-GMP in ΔXII strain attenuation, primary murine bone marrow-derived macrophages (BMDMs) were isolated from wild-type C57BL/6 and *STING*$^{gt/gt}$ (*STING*$^{-/-}$) mice and infected with the wild type *S.* Enteritidis strain 3934 or its derivative, ΔXII strain. Bacterial uptake and accumulation of IFN-β-mRNA was analyzed two and four hours post infection. Results showed that both strains were phagocytosed at similar rates and survived equally in wild type and *STING*$^{-/-}$ macrophages, indicating that neither the absence of c-di-GMP nor STING interfere with bacterial phagocytosis and survival (Fig. 2A). Two hours post infection, both strains induced significantly higher levels of IFN-β-mRNA in wild-type than in *STING*$^{-/-}$ BMDMs (Fig. 2B). These results suggested that induction of IFN-β production by *Salmonella* wild type and ΔXII strains was due to exposure of DNA in the cytosol and also that endogenous levels of c-di-GMP were not a contributor in triggering this response during macrophage infection.

Since c-di-GMP has also been shown to stimulate the STING-dependent induction of TNF-α when used as a nasal mucosal adjuvant

(Blaauboer *et al.*, 2014) and TNF-α together with other cytokines play a critical role in controlling *Salmonella* infection and the rate of growth (Dougan *et al.*, 2011), we then analyzed the STING mediated TNF-α production in the samples described above. *Salmonella* wild type and ΔXII strains induced high levels of TNF-α-mRNA both in wild-type and in *STING*$^{-/-}$ BMDMs (Fig. 2B), indicating that this TNF-α induction was related neither to STING nor to c-di-GMP.

Altogether, these results suggest that a *Salmonella* infection triggers induction of IFN-β production in macrophages that is independent of the presence of c-di-GMP and specific to the STING/TBK1/IRF3 pathway.

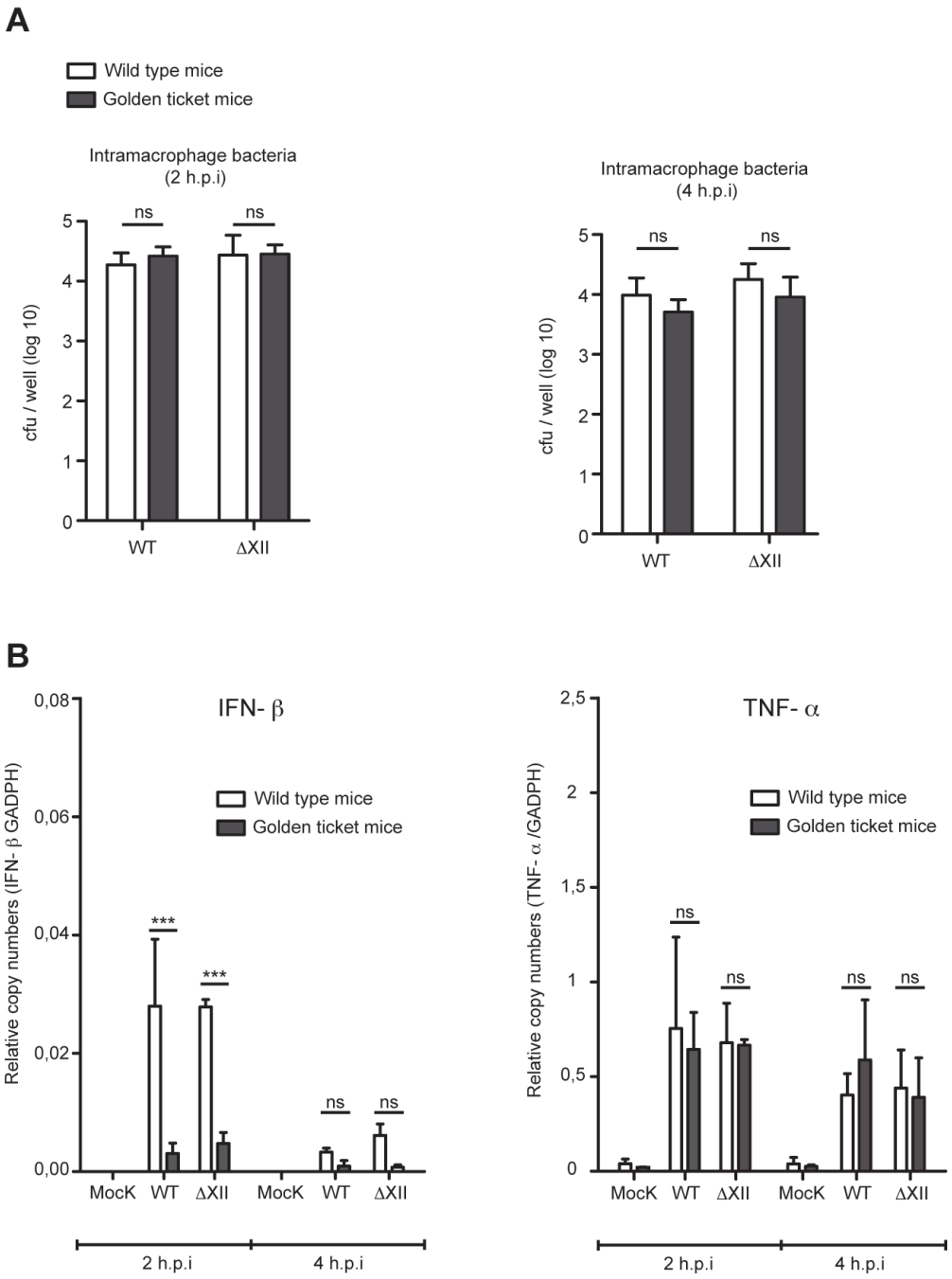

Fig. 2. Macrophage infection with *Salmonella* triggers induction of IFN-β production independently of c-di-GMP. A) WT and STING deficient bone marrow derived macrophages (BMDMs) were infected with *Salmonella* wild type and ΔXII strains at a multiplicity of infection (MOI) of 10 for 2 and 4 h and CFU were determined. B) Relative levels of expression of INF-β and TNF-α mRNA by WT and STING deficient BMDMs 2h and 4h postinfection with wild type and ΔXII strains. Mock representes cytokine levels of uninfected cells as a control. (n = 3 independent experiments). Statistical significance was determined using a two-way analysis of variance combined with the Bonferroni test. *P< 0.05; **P< 0.01; ***P<0.001.

ΔXII mutant attenuation is not a result of c-di-GMP/STING recognition

To further study how *Salmonella* and host cells use c-di-GMP and STING as a virulence factor and receptor of the innate immune system, respectively, we evaluated *Salmonella* wild type and ΔXII strains virulence in wild-type C57BL/6J and *STING*$^{-/-}$ mice.

Salmonella infections are usually initiated by ingestion of contaminated food or water followed by the passage of the bacteria from the stomach to the intestine. There, bacteria adhere and invade intestinal epithelial cells. Thus, we firstly compared the interaction of wild type and ΔXII strains with C57BL/6J and *STING*$^{-/-}$ mice intestinal epithelial cells, using the ligated ileal loop infection model (Solano *et al.*, 2009; Latasa *et al.*, 2016). The wild type strain showed a similar capacity to adhere and invade the intestinal epithelium of wild type and *STING*$^{-/-}$ mice, indicating that STING does not play a relevant role in the host during this step of infection. Accordingly, the ΔXII strain also invaded equally intestinal epithelial cells of wild type and *STING*$^{-/-}$ mice (Fig. 3).

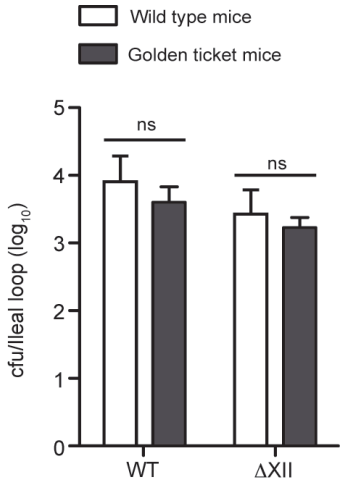

Fig. 3. The c-di-GMP/STING pathway does not play a significant role in the capacity of *Salmonella* to adhere and invade the intestinal epithelium. Adhesion and internalization rates of wild type and ΔXII strains using ileal loop infection experiments in wild type C57BL/6J (white bars) and *STING*$^{-/-}$ (black bars) mice. Seven ileal loops of each type of mouse were infected with 1x10^{7} cfu of wild type or ΔXII strains. Differences between wild type and *STING*$^{-/-}$ ileal loop colonization were statistically analysed by using a two-way analysis of variance combined with the Bonferroni test. *P< 0.05; **P< 0.01; ***P<0.001.

After penetrating the epithelial barrier, *Salmonella* preferentially infects naturally phagocytic cells in the mouse, such as dendritic cells and/or macrophages, within the lamina propia that favor dissemination through the lymphatics and blood to visceral organs: the mesenteric lymph nodes, spleen, and liver (Dougan *et al.*, 2011). Therefore, we then performed intraperitoneal infections with the wild type or ΔXII strains in wild-type C57BL/6 and *STING*$^{-/-}$ mice and addressed colonization at systemic sites by evaluating organ colonization. Both the wild type and ΔXII strains similarly colonized the spleen and liver of wild type and *STING*$^{-/-}$ mice (Fig. 4A). Finally, and in order to determine whether STING is involved in the colonization ability of *Salmonella* after infection by a natural route, we performed oral infections of wild-type C57BL/6 and *STING*$^{-/-}$ mice with the wild type or ΔXII strains and determined the number of infecting bacteria in the spleen, liver and caecum. Again, both strains colonized to the same extent the caecum and deeper tissues of wild-type and *STING*$^{-/-}$ mice (Fig. 4B).

Taken together, these findings indicated that host sensing of either DNA or c-di-GMP of *Salmonella* through the STING protein does not seem to play a significant role in the outcome of infection and hence, that ΔXII attenuation is not a result of STING-dependent host IFN responses.

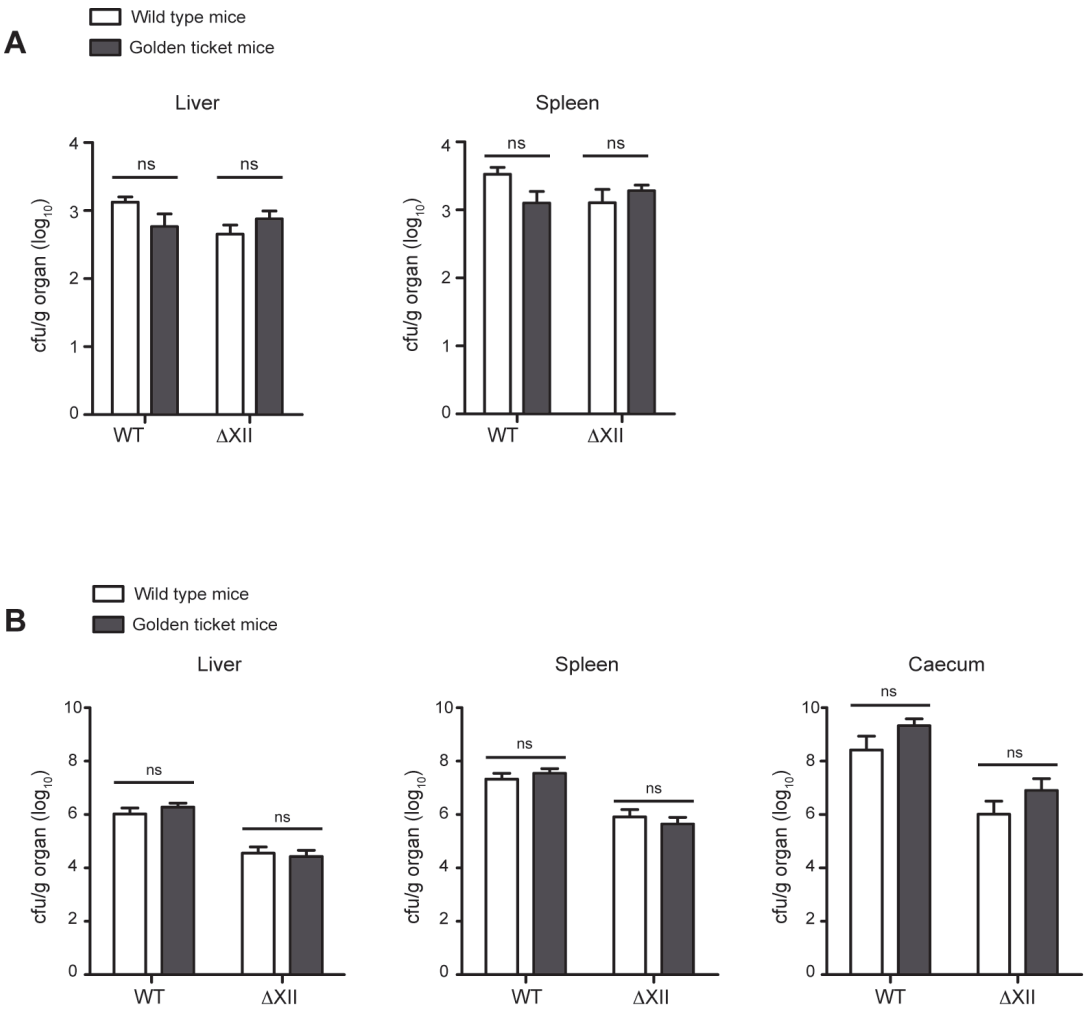

Fig. 4. The c-di-GMP/STING pathway is not involved in *Salmonella* organ colonization of i.p. and orally infected mice. (A) Colonization and invasiveness of livers and spleens from wild type C57BL/6J (white bars) and *STING*$^{-/-}$ (black bars) mice i.p. inoculated with 1x10^{4} cfu/mouse of wild type or ΔXII strains. Bacterial counts were performed 72 h after infection. (n = 6). (B) Colonization and invasiveness of spleens, livers and caecum from wild type C57BL/6J (white bars) and *STING*$^{-/-}$ (black bars) mice orally inoculated with 1x10^{8} cfu/mouse of wild type or ΔXII strains. Bacterial counts were performed 5 days after infection. (n = 6). Differences between wild type and *STING*$^{-/-}$ mice colonization were statistically analyzed by using a two-way analysis of variance combined with the Bonferroni test. *P< 0.05; **P< 0.01; ***P<0.001.

DISCUSSION

In a previous study we showed that a *Salmonella* multiple mutant, named ΔXII, deficient in the synthesis of c-di-GMP, presents a moderate attenuation in a systemic murine infection model (Latasa *et al.*, 2016), indicating that c-di-GMP signaling is needed for the development of an acute infection by a wild type strain. Based on these findings, in the current study we tried to elucidate whether c-di-GMP recognition by STING and the subsequent induction of type I interferons, contributes to *Salmonella* pathogenicity and is therefore related to ΔXII mutant attenuation. We thus considered the hypothesis that binding of c-di-GMP, synthesized by a wild type strain during infection, to the STING protein might induce the production of type I interferons. It is important to note that this is the first work that addresses the contribution of the c-di-GMP synthesized by *Salmonella* in triggering an innate immune response via STING during *in vitro* and *in vivo* infection processes.

Our results showing that a *Salmonella* wild type strain induces significantly higher levels of IFN-β-mRNA in wild-type than in *STING*$^{-/-}$ BMDMs supported our hypothesis. However, infection of macrophages with ΔXII mutant led to similar results indicating that *Salmonella* DNA rather than c-di-GMP activates the STING pathway in murine macrophages. These results contrasts with several studies that show a positive correlation between c-di-NMPs levels and IFN-I expression during *in vitro* macrophage infection. In this respect, it has been proven that c-di-AMP from *Listeria monocytogenes* (Woodward *et al.,* 2010; Sauer *et al.*, 2011; Jin *et al.*, 2011; Watson *et al.*, 2015), *Chlamydia trachomatis* (Barker *et al.*, 2013) and *Mycobacterium tuberculosis* (Dey *et al.*, 2015) and also c-di-GMP from *Legionella pneumophila* (Abdul-Sater *et al.*, 2012) activates a host type I interferon response in murine cells. With respect to *Salmonella*, our results differ from a previous work that demonstrated that macrophage infection

with *Salmonella enterica serovar* Typhimurium led to normal levels of IFN-β mRNA in *STING*$^{-/-}$ BMDMs, indicating that *Salmonella* predominantly activates type I IFNs via a STING independent mechanism, likely via the TLR4/TRIF pathway (Watson *et al.*, 2015).

While type I IFNs are potent antiviral signaling molecules, the role that they play during a bacterial infection is less clear and appears to depend on the bacterial species (Stetson and Medzhitov, 2006; Eshleman and Lenz, 2014). IFN-β expression protects against *Legionella pneumophila* (Schiavoni *et al.*, 2004; Plumlee *et al.*, 2009), *Chlamydia trachomatis* (Vignola *et al.*, 2010), group B streptococci, pneumococci, and *Escherichia coli* (Mancuso *et al.*, 2007), whereas promotes infection of several intracellular bacteria, including *Listeria monocytogenes* (Auerbuch *et al.*, 2004; Carrero *et al.*, 2004; O'Connell *et al.*, 2004) and *Francisella novicida* (Henry *et al.*, 2010). In the case of *Mycobacterium tuberculosis*, there are conflicting reports as to whether the IFN-β response is beneficial or detrimental to the host during tuberculosis (Manca *et al.*, 2001; Stanley *et al.*, 2007; Dey *et al.*, 2015). In most of these cases, the contribution of the c-di-NMPs-STING pathway to the overall IFN-β response to the *in vivo* bacterial infection remains unclear. STING is required for the type I IFN response to *L. monocytogenes in vivo* (Sauer *et al.*, 2011). However, STING deficiency in *STING*$^{-/-}$ and STING-knockout mice did not impact bacterial burdens in infected mice (Sauer *et al.*, 2011; Jin *et al.*, 2011). C-di-AMP also mediates IFN-β induction during *Mycobacterium tuberculosis* infection. Such an induction requires STING signaling and is independent of cGAMP. The enhanced levels of IFN-β activated by a *Mycobacterium tuberculosis* strain that secretes excess c-di-AMP leads to substantial virulence attenuation in mice (Dey *et al.*, 2015). In the case of *Salmonella*, the induction of type I interferon and its role in infection appear to depend on the route of entry

(Owen *et al.*, 2016). Intravenous infection with *S*. Typhimurium elicits a robust type I IFN response which drives necroptosis of macrophages and a compromised innate immune response (Robinson *et al.*, 2012). In contrast, oral infection induces barely detectable IFN-β expression in mice because *Salmonella* suppresses the TRIF-dependent Type I interferon response in macrophages as a virulence strategy (Owen *et al.*, 2016). Our *in vitro* data that suggest that IFN-β is produced in wild type BMDMs in response to a *Salmonella* infection do not correlate with *in vivo* results showing that a wild type *Salmonella* strain colonizes equally wild type and *STING*$^{-/-}$ mice, regardless of the route of entry. Therefore, our data establish that even if induction of IFN-β via STING actually occurred *in vivo*, the STING/TBK1/IRF3 pathway is not involved in *Salmonella* pathogenesis.

A recent study has shown that *Salmonella* makes cellulose inside macrophages (Pontes *et al.*, 2015) providing evidence of c-di-GMP production inside the cell, since cellulose synthesis is absolutely dependent on the allosteric activation by this dinucleotide. However, our results indicate that c-di-GMP does not play a role in modulating the innate immune response since both the wild type strain and ΔXII mutant colonized similarly wild type and *STING*$^{-/-}$ mice. This might be explained by the fact that there is no proof of c-di-GMP release from infecting bacteria to the cytosol that would allow its binding to STING. On the other hand, similar colonization of wild type and *STING*$^{-/-}$ mice by the wild type strain and ΔXII mutant also indicated that ΔXII mutant attenuation is not due to the lack of c-di-GMP sensing by STING. Our future studies will explore the reasons behind this attenuation.

In conclusion, analyses of the deviation of the immune response through c-di-NMPs accumulation in the host cell and of the consequences in the outcome of infection are still very incipient and the results seem to largely depend on the bacterial species and the type of c-di-NMP under

study. In the case of *Salmonella*, c-di-GMP binding to its eukaryotic receptor STING does not control the outcome of infection and thus, c-di-GMP does not seem to be an appropriate target for the treatment and prevention of *Salmonella* diseases.

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CHAPTER III

Salmonella lost the PGA exopolysaccharide to favor survival in the host

SUMMARY

Many bacteria build biofilm matrices using a conserved exopolysaccharide named PGA or PNAG (poly-β-1,6-*N*-acetyl-D-glucosamine). Interestingly, while *E. coli* and other members of the family *Enterobacteriaceae* encode the *pgaABCD* operon responsible for PGA synthesis, *Salmonella* lacks it. The evolutionary force driving this difference remains to be determined. Here, we report that *Salmonella* lost the *pgaABCD* operon after the divergence of *Salmonella* and *Citrobacter* clades, and previous to the diversification of the currently sequenced *Salmonella* strains. Reconstitution of the PGA machinery endows *Salmonella* with the capacity to produce PGA in a cyclic dimeric GMP (c-di-GMP) dependent manner. Outside the host, the PGA polysaccharide does not seem to provide any significant benefit to *Salmonella*: resistance against chlorine treatment, ultraviolet light irradiation and phage infection remained the same as in a strain producing cellulose, the main biofilm exopolysaccharide naturally produced by *Salmonella*. In contrast, PGA production proved to be deleterious to *Salmonella* survival inside the host, since it increased susceptibility to bile salts and oxidative stress, and hindered the capacity of *S*. Enteritidis to survive inside macrophages and to colonize extraintestinal organs, including the gallbladder. Altogether, our observations indicate that PGA is an antivirulence factor whose loss may have been a necessary event during *Salmonella* speciation to permit survival inside the host.

INTRODUCTION

Escherichia coli and *Salmonella enterica* are the two core species of the family *Enterobacteriaceae*, that constitutes a diverse group of bacteria that generally inhabit the gastrointestinal tract of animals. Although these two species are closely related, *E. coli* comprises commensal bacteria that do not normally cause disease, with the exception of certain pathogenic clones, whereas all members of *S. enterica* are considered pathogenic. Hence, an intriguing issue regarding bacterial evolution is the identification of determinants that make *Salmonella* able to establish parasitic interactions but enable *E. coli* to establish beneficial interactions with the human host. In this regard, it is believed that a combination of different genetic factors accounts for such a difference in virulence: first, *Salmonella* may harbor virulence genes that are not present in *E. coli*; second, *Salmonella* may have lost genes from the ancestral core genome that if present, would diminish its pathogenic potential; third, *E. coli* may carry a virulence suppressor gene(s) that interferes with the synthesis and/or stability of a virulence protein(s); and fourth, *Salmonella* and *E. coli* may differ in the regulation of cellular factors important for survival in the host (Ochman *et al.*, 2000; Bliven and Maurelli, 2012; Baumler and Fang, 2013).

An intriguing difference between *Salmonella* and *E. coli* that might account for their distinctive lifestyles as regards the human host is the exopolysaccharide that each species uses to build the biofilm matrix. Bacteria spend most of their lives inside a biofilm surrounded by a highly hydrated layer that provides protection against desiccation, diffusion of antibiotics, toxic metal ions and other compounds, predation by protozoans and the host immune system, amongst others (Costerton *et al.*, 1987; Flemming and Wingender, 2010). Diversity in biofilm exopolysaccharides composition is high, with some bacterial species being able to produce different types depending on the environmental conditions (Lembré *et al.*,

2012; Flemming *et al.*, 2016). In parallel to such high diversity and for reasons that remain unknown, a wide range of phylogenetically distant bacteria make use of the same exopolysaccharide to embed themselves inside a biofilm. One example of a "universal" exopolysaccharide is cellulose, composed of β(1-4)-linked D-glucose units, used by a wide variety of bacteria, including both *E. coli* and *Salmonella* (Zogaj *et al.*, 2001; Solano *et al.*, 2002; Römling and Galperin, 2015), as a significant biofilm matrix component. Another example corresponds to a homopolysaccharide composed of N-acetylglucosamine with β(1–6) glycosidic linkage (Cywes-Bentley *et al.*, 2013). Production of this exopolysaccharide was firstly described in *Staphylococcus epidermidis and S. aureus* where it was referred to as PIA/PNAG (Mack *et al.*, 1996; McKenney *et al.*, 1998; Cramton *et al.*, 1999). Later on, the synthesis of a similar exopolysaccharide was also reported in *E. coli* where it was named as PGA (Wang *et al.*, 2004), and also in *Acinetobacter baumannii* (Choi *et al.*, 2009), *Klebsiella pneumonia* (Chen *et al.*, 2014), *Bordetella bronchiseptica* and *B. pertussis* (Sloan *et al.*, 2007; Conover *et al.*, 2010), *Actinobacillus pleuropneumoniae* (Izano *et al.*, 2007), *Yersinia pestis* (Bobrov *et al.*, 2008), *Burkholderia* species (Yakandawala *et al.*, 2011) and *Bacillus subtilis* (Roux *et al.*, 2015). In these bacteria, several functions have been ascribed to PGA such as surface attachment, intercellular adhesion, biofilm formation, epithelial cell attachment, and resistance to antibiotics, antimicrobial peptides and human PMNs (Vuong, Kocianova, *et al.*, 2004; Maira-Litran *et al.*, 2005; O'Gara, 2007; Izano *et al.*, 2008; Choi *et al.*, 2009; Conover *et al.*, 2010; Bobrov *et al.*, 2010; Yakandawala *et al.*, 2011; Bentancor *et al.*, 2012). In *E. coli*, the production, modification, and export of PGA requires the machinery encoded by the *pgaABCD* operon (Wang *et al.*, 2004). PgaA and PgaB are needed for poly-GlcNAc export and PgaC and PgaD are necessary for poly-

GlcNAc synthesis (Itoh *et al.*, 2008; Little *et al.*, 2012; Whitney and Howell, 2013; Steiner *et al.*, 2013). As it generally occurs for bacterial exopolysaccharides, PGA synthesis is allosterically activated by the second messenger bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) (Steiner *et al.*, 2013; Romling *et al.*, 2013; Pérez-Mendoza and Sanjuán, 2016). Strikingly, *Salmonella* lacks the *pgaABCD* operon and any identifiable genetic loci similar to *pga* required for PGA synthesis.

Here, we pursue the reasons that explain why *E. coli* and *Salmonella* differ in their capacity to produce the PGA exopolysaccharide. We provide evidence that production of PGA reduces *Salmonella* resistance against bile salts and its capacity to survive inside macrophages, completely impairing the infection cycle and rendering *Salmonella* avirulent. Together, these observations highlight the relevance of gene loss in the adaptation to novel pathogenic niches and define the loss of the PGA exopolysaccharide as a landmark event during *Salmonella* speciation.

MATERIALS AND METHODS

Phylogenetic analyses

Protein sequences from *E. coli* PgaABCD and PhoH were used in a Blastp search against the NCBI non-redundant database accessed in July 2016, using an e-value threshold of 10^{-5} and excluding from the results hits taxonomically assigned to *E. coli*. The sequences from the top 500 hits were retrieved for each search and aligned using MUSCLE v 3.8 (Edgar, 2004) and then trimmed using trimAl v1.4 (Capella-Gutiérrez *et al.*, 2009) (gap-score cut-off 0.9). A Maximum Likelihood phylogenetic reconstruction was performed using phyML v3.0 (Guindon *et al.*, 2010) with the JTT model, setting the number of rate categories to four, and inferring the number of invariant positions and the parameters of the gamma distribution from the data. Branch support was computed using an aLRT (approximate likelihood ratio test) based on a chi-square distribution.

Ethics statement

Animal studies were performed in accordance with the European Community guiding in the care and use of animals (Directive 2010/63/EU). Protocols were approved by the ethics committee of the Public University of Navarra (Comité de Ética, Experimentación Animal y Bioseguridad of the Universidad Pública de Navarra) (approved protocol PI-004/11). Work was carried out in the animal facility of the Instituto de Agrobiotecnología, Universidad Pública de Navarra. Animals were housed under controlled environmental conditions with food and water ad libitum. Mice were euthanized by CO_2 inhalation followed by cervical dislocation and all efforts were made to minimize suffering.

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this work are described in S1 Table. *Escherichia coli* and S. *enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) cells were grown in LB broth and on LB agar (Pronadisa) with appropriate antibiotics at the following concentrations: kanamycin (Km), 50 μg ml^{-1}; ampicillin (Am), 100 μg ml^{-1}; carbenicillin (Cb), 50 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; and streptomycin (Sm) 500 μg ml^{-1}.

DNA manipulations

Routine DNA manipulations were performed using standard procedures unless otherwise indicated. Plasmid DNA from *E. coli* was purified using a Quantum Prep plasmid kit (BioRad). Plasmids were transformed into *E. coli* and *S.* Enteritidis by electroporation. Transformants carrying Red helper plasmids were made electro-competent as described (Solano *et al.*, 2002; Blank *et al.*, 2011). Restriction enzymes were purchased from ThermoFisher Scientific and used according to the manufacturer's instructions. Oligonucleotides were synthesized by StabVida (Caparica—Portugal) and are listed in S2 Table. Phage P22 HT105/1 int-201 (Schmieger, 1972) was used to carry out transductions between strains according to recommended protocols (Maloy and Nunn, 1981).

Construction of a collection of strains containing a single GGDEF domain protein

S. Enteritidis 3934 ΔXII is a multiple mutant carrying mutations in all genes encoding GGDEF domain proteins (Zorraquino *et al.*, 2013). Derivatives of ΔXII containing the following single GGDEF protein encoding gene, namely *adrA*, *yeaJ*, *sen1023*, *yciR, yegE*, *yfiN*, *yhdA*, *sen3222*, and *yhjK* were constructed as described (Solano *et al.*, 2009). In the case of ΔXII+*sen2484*, ΔXII+*yfeA* and ΔXII+*sen4316* strains, DNA fragments

corresponding to the coding sequences of *sen2484*, *yfeA* and *sen4316 genes* were amplified with primer pairs A and D and chromosomal DNA from *S.* Enteritidis 3934 as a template. Amplified fragments were sequenced and cloned into the pKO3blue plasmid that was electroporated into ΔXII. Integration and excision of the plasmid was performed as described (Solano *et al.*, 2009) in order to obtain the corresponding restored strains.

Chromosome expression of *adrA* under PcL and P_{phoP} promoters

To express *adrA* under the PcL constitutive promoter in *S.* Enteritidis 3934, a PCR generated linear DNA fragment was used as described (Datsenko and Wanner, 2000) with some modifications. The Red helper plasmid pKD46 was transformed into *S.* Enteritidis 3934, and transformants were selected on LB agar Am after incubation at 30ºC for 24 h. One transformant carrying pKD46 was made electrocompetent as described (Solano *et al.*, 2002). A DNA fragment containing a kanamycin resistance gene, the PcL promoter and the RBS sequence of the PcL cassette was generated by PCR using primers *adrA* Km PcL rbs Fw and *adra* Km PcL rbs Rv and chromosomal DNA from strain MG1655 Km PcL-λATT-GFP as template (Da Re *et al.*, 2007). Electroporation (25 mF, 200 W, 2.5kV) was carried out according to the manufacturer's instructions (Bio-Rad) using 50 μl of cells and 1 to 5 μg of purified and dialysed (0.025 μm nitrocellulose filters; Millipore) PCR product. Shocked cells were added to 1 ml of LB broth, incubated for 1 h at 28ºC and then spread on LB Km agar to select Km^R transformants after incubation at 37ºC for 24 h. Transformants were then grown on LB Km broth at 44ºC for 24 h and incubated overnight on LB Am agar at 28ºC to test for loss of the helper plasmid.

To place the *adrA* gene under the control of the *phoP* promoter, a protocol described previously was carried out with some modifications (Blank *et al.*,

2011). In a first step, primers Km SceI P_{phoP} *adrA* Fw and Km SceI P_{phoP} *adrA* Rv, with 60-bp homology extensions, were used to amplify a kanamycin resistance cassette and an I-SceI recognition site from plasmid pWRG717. This DNA was integrated upstream the *adrA* gene via λ Red-mediated recombination using plasmid pWRG730, a temperature-sensitive plasmid for independent inducible expression of the λ Red recombinase and I-SceI endonuclease. After confirming proper insertion of the resistance cassette by colony PCR with primers 01-E and Km SceI P_{phoP} *adrA* Rv, a DNA fragment generated by PCR and derived from oligonucleotides P_{phoP} *adrA* Fw and P_{phoP} *adrA* Rv and *S.* Enteritidis 3934 chromosomal DNA as template, was electroporated into the mutant strain still containing the pWRG730 plasmid. This DNA fragment included the *phoP* promoter and homology regions used for its upstream *adrA* integration. After 1 h of incubation at 28°C, 100 µl of a 10^{-2} dilution was plated on LB agar plates containing 500 ng ml^{-1} anhydrotetracycline, which induced expression of I-SceI endonuclease. After overnight incubation at 28°C, single colonies were purified and successful recombination was checked by monitoring absence of antibiotic resistance, colony PCR with oligonucleotides 01-E and P_{phoP} *adrA* Rv, and sequencing of the resulting fragment. Finally, pWRG730 was cured by incubating selected colonies at 44°C.

Construction of a *Salmonella* strain that constitutively expresses the *pgaABCD* operon from the chromosome

To insert the *pgaABCD* genes from *E. coli* K-12 MG1655 into the *S.* Enteritidis 3934 chromosome, the T64B prophage site was chosen (Mmolawa *et al.*, 2003). Two DNA fragments, *sb13* AB and *sb13* CD, of ~500 bp length of the *S.* Enteritidis *sb13* gene, were amplified with primer pairs SmaI *sb13* AB Fw/SphI *sb13* AB Rv and SphI *sb13* CD Fw/SalI *sb13*

CD Rv, respectively. The PCR products were cloned into the pJET 1.2 vector (ThermoFisher Scientific) and resulting plasmids were digested with SmaI and SphI enzymes in the case of the AB fragment and SphI and SalI enzymes in the case of the CD fragment. AB and CD fragments were ligated in the same ligation mixture with the pKO3 vector (Link *et al.*, 1997) digested with SmaI and SalI enzymes, resulting in plasmid pKO3::*sb13*AD. The pJET::*pga* plasmid constructed in this study was digested with SphI to obtain a DNA fragment containing the *pga* promoter and *pgaABCD* genes. P_{pga}::*pgaABCD* was ligated with pKO3::*sb13*AD digested with SphI, resulting in pKO3::*sb13*AD-P_{pga}::*pgaABCD* plasmid. Integration and excision of the plasmid was used as described (Link *et al.*, 1997) to obtain WT P_{pga}::*pgaABCD* strain. Insertion of P_{pga}::*pgaABCD* into the *sb13* gene was confirmed by PCR using primers *sb13* OK Fw and *pgaA* comp Rv. The ability of this strain to produce PGA was not detectable by Dot Blot, probably because heterologous chromosomal expression of the *pgaABCD* operon under its own promoter was not sufficient to produce evident PGA levels. Thus, a second *Salmonella* strain was generated in order to express *pgaABCD* under the PcL constitutive promoter and in the chromosome. To do so, a 427 bp DNA fragment, namely *sb13* AB_2, of the *S.* Enteritidis *sb13* gene was amplified with primers BglII *sb13* AB Fw and BamHI sb13 AB Rv, using *S.* Enteritidis 3934 chromosomal DNA as template, and cloned into the pJET 1.2 vector (ThermoFisher Scientific). A second DNA fragment containing the PcLrbs promoter (Da Re *et al.*, 2007) and the first 543 bp of the *pgaA* gene coding sequence was constructed by overlapping PCR, using two separate PCR products. Primers BamHI PcLrbs Fw and sb13 PcL*pga* Rv were used to amplify the PcLrbs promoter, using *E. coli* MG1655 Km PcL-λATT-GFP chromosomal DNA as template (Da Re *et al.*, 2007). Primers PcL *pgaA* Fw and PstI PcL *pgaA* Rv were used to amplify 543 bp of the *pgaA* gene, using *E. coli* MG1655 chromosomal DNA as template.

These two purified PCR products were mixed, and a second PCR using BamHI PcL rbs Fw and PstI PcL *pgaA* Rv primers was performed to obtain a single DNA fragment, PcLrbs::*pgaA*, that was cloned into the pJET 1.2 vector (ThermoFisher Scientific). Plasmids pJET::sb13AB_2 and pJET:: PcLrbs::*pgaA* were digested with BglII /BamHI and BamHI/PstI enzymes, respectively, and digestion products were ligated in the same ligation mixture with the pKO3Blue vector (Solano *et al.*, 2009) digested with BglII and PstI enzymes, resulting in plasmid pKO3Blue::*sb13*AB_2-PcLrbs::*pgaA* that was electroporated in WT P_{pga}::*pgaABCD* strain. Integration and excision of the plasmid was used as described (Solano *et al.*, 2009) to generate Wt PcLrbs::*pgaABCD*. Insertion of PcLrbs::*pgaABCD* into the *sb13* gene was confirmed by PCR using primers *sb13* OK Fw and sb13 PcL *pgaA* Rv. Finally, a *bcsA* mutation was transduced from Δ*bcsA* strain to generate Δ*bcsA*::Cm^R PcLrbs::*pgaABCD*, which is hereafter abbreviated as Δ*bcsA* PcL::*pga*.

Construction of an *E. coli* strain that constitutively expresses the *pgaABCD* operon from the chromosome

To express the *pgaABCD* operon under the PcL promoter in *E. coli* MG1655, a PCR generated linear DNA fragment and the Red helper plasmid pKD46 were used as described above. Primers used to generate the DNA fragment containing a kanamycin resistance gene, the PcL promoter and the RBS sequence of the PcL cassette were Km PcL rbs *pga* Fw and Km Pcl rbs *pga* Rv.

Construction of an *E. coli* MG1655 Δ*pgaC* mutant

To delete a 500 bp fragment of the *pgaC* gene in *E. coli* MG1655, and as a consequence suppress PGA production in *E.coli* (Wang *et al.*, 2004), a

protocol described previously was carried out with some modifications (Blank *et al.*, 2011). First, primers *pgaC* Km SceI Fw and *pgaC* Km SceI Rv, with 60-bp homology extensions, were used to amplify a kanamycin resistance cassette and an I-SceI recognition site from plasmid pWRG717. This DNA was integrated in the *pgaC* gene using plasmid pWRG730 plasmid and integration was confirmed by colony PCR with primers *pgaC* Km SceI Fw and *pgaD* Rv. Phosphorylated 80-mer double-stranded DNA derived from oligonucleotides Δ*pgaC* Fw and Δ*pgaC* Rv was electroporated into the mutant strain still containing the pWRG730 plasmid. After 1 h of incubation at 28°C, 100 µl of a 10^{-2} dilution was plated on LB agar plates containing 500 ng ml^{-1} anhydrotetracycline, which induced expression of I-SceI endonuclease. After overnight incubation at 28°C, single colonies were purified, and successful recombination was checked by monitoring absence of antibiotic resistance and colony PCR with oligonucleotides Δ*pgaC* Fw and *pgaD* Rv. Finally, pWRG730 was cured by incubating selected colonies at 44°C.

PGA quantification

PGA exopolysaccharide levels were quantified as previously described (Cramton *et al.*, 1999) with minor modifications. Briefly, cultures in 5 ml LB or LB Am broth of the strains tested were adjusted to the same number of cells and centrifuged at 18,000 x g for 5 min. Pellets were resuspended in 50 µl of 0.5 M EDTA (pH 8.0) and suspensions were incubated for 5 min at 100°C and centrifuged at 18,000 x g for 5 min. Each supernatant (40 µl) was incubated with 10 µl of proteinase K (20 mg ml^{-1}) (Sigma) for 30 min at 37°C. After the addition of 10 µl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 5 µl were spotted on a nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-

Rad). The membrane was blocked overnight with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween 20, and incubated for 2 h with specific anti-PNAG antibodies diluted 1:10,000 (Maira-Litran *et al.*, 2005). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch Laboratories, Inc., West- grove, PA) diluted 1:10,000 and developed using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). All extracts assayed in a particular experiment were analyzed on the same membrane. Images obtained in a GBox Chemi HR16 system (Syngene) were cut and put together to assemble horizontal figures showing PGA quantification.

Biofilm formation

The cellulose mediated biofilm formed in glass tubes on standing rich cultures was examined visually after growth in 5 ml of LB broth at room temperature for 72 h (Solano *et al.*, 2002). The PGA mediated biofilm was visualized after growth in LB broth at 28ºC in an orbital shaker (250 r.p.m) for 16 h (Valle *et al.*, 2003). Macrocolony biofilms formed on the surface of LB agar plates were formed after spotting 50 µl drops of overnight liquid cultures and incubating at 28ºC for 48 hours (Serra, Richter, Klauck, *et al.*, 2013).

Scanning electron microscopy

For scanning electron microscopy bacterial strains were grown under biofilm forming conditions. Growth medium was removed and bacterial cells were fixed by adding a fixation solution (1.3% glutaraldehyde, 0.07M cacodylate buffer and 0.05% rhutenium red). Samples were then washed in and post-fixed by incubation with 2% osmium tetroxide for 1 h. Bacteria were then fully dehydrated in a graded series of ethanol solutions and dried

in hexamethyldisilazane (HMDS, Sigma). Finally, samples were coated with 40 Å platinum, using a GATAN PECS 682 apparatus (Pleasanton, CA), before observation under a Zeiss Ultra plus FEG-SEM scanning electron microscope (Oberkochen, Germany) (Laboratoire de Biologie Cellulaire et Microscopie Electronique, UFR Médecine (Tours, France)).

Hydrophobicity assay

Macrocolony biofilms were formed on the surface of LB or LB Cb agar plates as described above, and a 10 µl water droplet stained with red food colouring was placed on the biofilm to show the hydrophobicity exhibited by the structure (Cairns *et al.*, 2014).

Sodium hypochlorite treatment of macrocolony biofilms

To perform sodium hypochlorite survival analyses, a protocol described previously was carried out with some modifications (Solano *et al.*, 2002). Macrocolony biofilms were formed on the surface of LB or LB Cb agar plates as described above and then treated with 10 ml PBS containing 200 p.p.m. sodium hypochlorite for 40 min at 37ºC. Control samples were incubated with 10 ml of PBS. Macrocolonies were harvested with a bent tip and bacteria were washed in PBS three times and suspended in 5 ml of PBS. After vortexing and sonicating (30 sec; potency 3; Branson sonifier 250; microtip), bacteria were enumerated by viable plate counts.

UV light treatment

Bacterial strains were grown in LB Cb broth at 28ºC in an orbital shaker (200 r.p.m) for 16 h. After sonication (30 sec; potency 3; Branson sonifier 250; microtip), the OD_{600nm} was adjusted to 1 and serial dilutions were plated on four plates of N media agar supplemented with Cb (Pontes *et al.*, 2015). After 24h of growth at 28ºC, two plates were irradiated with UV

light for 5 min. All plates were then incubated at 28ºC for 48h and the numbers of surviving bacteria were counted. Results are shown as % survival relative to non-irradiated samples. Experiments were conducted in triplicate.

In order to incubate all strains on the same plates, strains *ΔbcsA* PcL::*adrA* and WT PcL::*adrA* were transformed with a pJET empty plasmid.

Susceptibility of biofilms to phage infection

Overnight cultures in LB or LB Cb broth were sonicated (30 sec; potency of 3; Branson sonifier 250; microtip) and the OD_{600nm} was adjusted to 1. Sterile polymer membrane filters (diameter 47 mm; Millipore) were placed on LB or LB Cb agar plates and seeded with a 50 µl drop of each bacterial suspension. Plates were inverted and incubated at 28°C for 48 h to allow macrocolony biofilm formation on top of the filters, that were then transferred to an empty petri dish and treated with a P22 phage lysate generated from the streptomycin resistant strain *S.* Typhimurium SL1344. After 1h of incubation at 37ºC, the entire content of the plates was collected, washed in PBS and plated on LB Sm agar. The number of streptomycin resistant cfu were indicative of transduction efficiency. Experiments were conducted in triplicate.

Macrophage survival assay

Macrophage survival assay was conducted essentially as described (Pontes *et al.*, 2015) with some modifications. The murine macrophage cell line RAW 264.7 was propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin/Streptomycin/Glutamine (Gibco). Macrophages were seeded at a density of 2 x 10^5 cells per well in 24-well plates 24 h prior to infection. *Salmonella* overnight cultures grown at 37ºC in LB broth were

sonicated (30 sec; potency 3; Branson sonifier 250; microtip) and diluted 1:100 in LB broth. Cultures were incubated at 37ºC in an orbital shaker (200 r.p.m) to an OD_{600nm} of 1 and the suspension was sonicated again and washed twice with DMEM deprived of serum. Macrophages were then infected with *Salmonella* strains at a multiplicity of infection of approximately 10:1 and plates were centrifuged at 1000 r.p.m. for 10 minutes at room temperature. After 20 min of phagocytosis, monolayers were washed twice with PBS and treated with gentamicin (100 µg ml^{-1}) for 1 h. To estimate phagocytosed bacteria, samples were then washed three times with sterile PBS and macrophages were lyzed with 1% (vol/vol) Triton X-100-PBS to release intracellular bacteria that were counted by plating 25 µl of serial dilutions onto LB plates. To assess bacterial survival, medium was replaced by DMEM supplemented with 10% FBS and 12 µg ml^{-1} gentamycin and the cells were incubated at 37ºC. After 18 h of infection, wells were washed twice with PBS and were treated with Triton X-100 as indicated above. The percentage survival was obtained by dividing the number of bacteria recovered after 18 h by the number of phagocytosed bacteria and multiplying by 100. At each stage when infected cells were lysed, the number of viable cells in duplicate monolayers infected with each strain was assessed by 0.4% trypan blue exclusion and counting viable cells. No difference in viability was noted between cells infected with the different strains. Experiments were done in triplicate on three independent occasions.

Polymyxin B resistance assay

One-hour polymyxin susceptibility assays were performed as described (Groisman *et al.*, 1997). Polymyxin B Sulfate (Sigma) was used at a final concentration of 2.5 µg ml^{-1}. Data are presented as survival

percentage relative to samples incubated in LB without polymyxin. Experiments were conducted in triplicate.

Hydrogen peroxide sensitivity assay

Sensitivity to hydrogen peroxide was tested as previously described (Bogomolnaya *et al.*, 2013) with minor modifications. Briefly, overnight cultures were subcultured at 1/100 in 5 ml LB containing either no or 1 mM H_2O_2 (Merk). Replica cultures were used for each time point. Cultures were grown at 37°C with aeration and collected hourly. LB broth contains ~30-40 µM Mg^{2+} (Maguire and Papp-Wallace, 2008), which activates the *phoP* promoter, thus, leading to *adrA* expression. After sonication (30 sec; potency 3; Branson sonifier 250; microtip) the number of surviving bacteria were counted by plating serial dilutions onto LB plates. Experiments were performed on three separate occasions.

Ileal loop co-infection experiment

In order to differentiate strains in all mice competitive infections performed, the wild type and *ΔbcsA* PcL::*pga* strains were made streptomycin (Sm) resistant through P22 phage transduction of the *aadA* gene from the natural streptomycin resistant strain *S.* Typhimurium SL1344 (Suar *et al.*, 2006).

To compare the *in vivo* interaction of *Salmonella* strains with murine intestinal epithelial cells, the ligated ileal loop co-infection model was used as described previously (Solano *et al.*, 2002; Latasa *et al.*, 2005). Strains were incubated on LB agar for 48 hours at room temperature, suspended in PBS and sonicated (30 sec; potency 3; Branson sonifier 250; microtip) prior to infection. Competitive index (CI) was defined as the $\log_{10}$ of the ratio of the exopolysaccharide producing strain to control strain recovered (Output)

divided by the ratio of the exopolysaccharide producing strain to control strain present in the inoculum (Input). A CI > 0 indicates the exopolysaccharide producing strain with a colonization advantage compared to the control and a CI < 0 indicates the exopolysaccharide producing strain with a colonization disadvantage over the control.

Colonization experiments

Colonization experiments were carried out with 8-week-old female BALB/c mice (Charles River Laboratories). Mice were acclimated for 7 days after arrival before the experiments were started in the animal facility of the Instituto de Agrobiotecnología, Universidad Pública de Navarra. Food and water were removed, twelve and two hours respectively, before the administration of bacterial suspension. Mice were prefed with 20 µl of 10% sodium bicarbonate 30 min before bacterial inoculation. Water and food were again supplied right after inoculation.

Strains were incubated on LB agar for 48 hours at room temperature, suspended in PBS and sonicated (30 sec; potency 3; Branson sonifier 250; microtip) prior to infection. Mice were inoculated intragastrically with 100 µl of bacterial suspensions. In the case of coinfection experiments, the total bacteria inoculum was 2 x 10^8 cfu of combined polysaccharide producing strain and *ΔbcsA* strain at a ratio of 1:1. In the case of individual infections, inoculum was 1 x 10^8 cfu of the strain analysed. The cfu of each strain in the inoculum (input) were quantified by plating dilution series on LB agar supplemented with chloramphenicol and LB agar supplemented with streptomycin to distinguish between strains. Over the course of infection, mice were examined twice per day and a final disease score was given to each mouse according to clinical signs observed as follows. No clinical signs (0); mild clinical signs: ruffled fur (1); moderate clinical signs: ruffled

fur plus, lethargy, hunched posture and decreased activity (2); severe clinical signs: paresis, paralysis, tremor, shivers, ataxia, rigidity (3). When evident signs of disease (score 2 to 3) were observed, mice were euthanized by CO_2 inhalation followed by cervical dislocation. Then, dilution series of liver, spleen and gallbladder lysates were plated on LB agar for enumeration of cfu (output), using antibiotic resistance to differentiate strains. Values for CI were calculated as described above.

Bile and SDS sensitivity assays

Bile bovine sensitivity assay was performed as described (Ramos-Morales *et al.*, 2003) with minor modifications. Bacterial strains were grown in LB broth at 28ºC for 48 h in an orbital shaker (200 r.p.m). Two microliter portions of serial dilutions were incubated for 24 h at 37°C in LB agar plates containing either no or bile bovine (Sigma). The bile concentration used was 24% or 13% when assessing *Salmonella* and *E. coli* bile sensitivity, respectively.

To carry out the SDS MIC analysis, bacterial strains were grown in LB broth at 28ºC for 48 h in an orbital shaker (200 r.p.m) and diluted such that samples of $2x10^3$ CFU/ml were subjected to various concentrations of SDS in polypropylene microtiter plates (ThermoFisher Scientific). The plates were incubated overnight at 37°C under nonaerated conditions and the wells of the plate were visually analyzed to determine the MICs.

Statistical analyses

All statistical analyses were performed in GraphPad Prism 5.01. Sodium hypochlorite survival, UV light irradiation data, susceptibility of biofilms to phage infection, macrophage survival and Polymyxin B resistance analyses were analysed by the Mann-Whitney *U* test. A two-way analysis of variance combined with the Bonferroni test was used to analyse

statistical significance in hydrogen peroxide sensitivity assays. A nonparametric Mann-Whitney *U* test and an unpaired Student's t test were used to assess significant differences in individual colonization or coinfection experiments, respectively.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	MIC[a]	Reference or source
Strains			
***S.* Enteritidis**			
3934	Wild-type clinical isolate	54	(1)
ΔXII	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	3664	(2)
Δ*bcsA*	3934 Δ*bcsA*::Cm^R	3702	(2)
WT PcL::*adrA*	3934 Km^R PcL rbs::*adrA*	4397	This study
Δ*bcsA* PcL::*adrA*	3934 Δ*bcsA*::Cm^R Km^R PcL rbs::*adrA*	5583	This study
WT P_{phoP}::*adrA*	3934 P_{phoP}::*adrA*	5624	This study
Δ*bcsA* P_{phoP}::*adrA*	3934 Δ*bcsA*::Cm^R P_{phoP}::*adrA*	5626	This study
Δ*bcsA* P_{phoP}::*adrA* PcL::*pga*	Δ*bcsA*::Cm^R P_{phoP}::*adrA* PcL rbs::*pga*	5860	This study
Δ*bcsA* PcL::*pga*	3934 Δ*bcsA*::Cm^R PcL rbs::*pgaABCD*	5735	This study
ΔXII + *adrA*	3934 Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2785	This study
ΔXII + *yeaJ*	3934 Δ*adrA* Δ*sen1023* Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2786	This study
ΔXII + *yciR*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2787	This study
ΔXII + *sen1023*	3934 Δ*adrA* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2788	This study
ΔXII + *yegE*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2789	This study

ΔXII + *yfeA*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* Δ*sen4316*	2790	This study
ΔXII + *yfiN*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2791	This study
ΔXII + *yhdA*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2792	This study
ΔXII + *sen3322*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2793	This study
ΔXII + *yhjK*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*sen2484* *yfeA*::Km^R Δ*sen4316*	2794	This study
ΔXII + *sen4316*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* Δ*sen2484* *yfeA*::Km^R	3831	This study
ΔXII + *sen2484*	3934 Δ*adrA* Δ*sen1023* *yeaJ*::Tc^R Δ*yciR* Δ*yegE* Δ*yfiN* Δ*yhda* Δ*sen3222* Δ*yhjK* *yfeA*::Km^R Δ*sen4316*	2795	This study
E.coli			
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdr*17 *supE*44 *rel*A1 *lac* [F´*proAB lacI*^{q} Z Δ*M15* Tn10 (Tc^R)]	797	Stratagene
K-12 MG1555	Wild type commensal strain	5359	Provided by J. M. Ghigo
MG1655 Km PcL-λATT-GFP	MG1655 Km^R PcL-λATT-GFP	3023	(3)
MG1655 Δ*pgaC*	MG1555 Δ*pgaC*	5487	This study
MG1655 PcL::*pga*	MG1555 Km^R PcL rbs::*pgaABCD*	5732	This study
Plasmids			
pJET 1.2	Cloning vector Am^R		Thermo Scientific

pKD46	Temperature-sensitive plasmid containing λ red recombinase genes under the control of an arabinose-inducible promoter; Am^R	(4)
pWRG717	Plasmid with I-SceI recognition site, Km^R. Use for kanamycin cassette amplification in rapid mutagenesis.	Provided by R. G. Gerlach
pWRG730	Plasmid with I-SceI endonuclease under control of tetracycline-inducible promoter (P_{tetA}), temperature-sensitive, Cm^R	Provided by R. G. Gerlach
pKO3	Cm^R vector for recombination experiments	(5)
pKO3Blue	Cm^R, derivative of pKO3 carrying the pMAD lacZ gene under the control of the P*clpB* promoter.	{CristinaSolanoa:2009uo}
pJET::*pga*	pJET 1.2 containing the *pgaABCD* operon of *E.coli* MG1655	This study
pKO3::*sb13*AD	pKO3 containing fragments AB and CD from the *sb13* gene of T64B phage.	This study
pKO3::*sb13*AD-P_{pga}::*pgaABCD*	pKO3 containing fragments AB and CD from the *sb13* gene of T64B phage and the *pgaABCD* operon of *E.coli* MG1655 under its own promoter	This study
pKO3Blue::*sb13*AB_2-PcLrbs::*pgaA*	pKO3Blue containing fragment AB_2 from the *sb13* gene of T64B phage and the *pgaA* gene under the PcLrbs promoter	This study
pCN40	Vector encoding ampicillin resistance	(6)

[a]Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra.

Table 2. Oligonucleotides used in this study.

Primer	Sequence 5´→3´
Cloning and sequencing of *pgaABCD* and *pgaABCD-ycdT* genes into the pJET vector	
pgaA Fw	**GCATGC**CCGAAATCATGCATCGGAATTTACTGATT
pgaD Rv	**GCATGC**TTATGCCCGGACTAGCGCTT
pgaA sec Fw	CCGCACTGCCCAGTACCA
pgaB sec Fw	GTGCGCGCGTAAAAGCCAT
pgaC sec Fw	GCCCTGGCAGAAGTGGGTTA
***adrA* chromosome expression under the PcL promoter**	
adrA Km PcL rbs Fw	CCGAAATTTCATACTTCCTCCATGCGCCCTGTTTCTATAATTTG GGAAAATTGTTTCTAATTCGCTCAAGTTAGTAATTCTCAC[a]
adrA Km PcL rbs Rv	TACCGCCTGTTCAACCGCTTTTCGGTAAAAATTTTCATCATTCA TTATTTTTGGGAACATGCGGTACCTTTCTCCTCTTTAATG[b]
***adrA* chromosome expression under the *phoP* promoter**	
Km SceI P_{phoP} *adrA* Fw	GTAGCAAGTTTATGAGCGCCTGCCTGAAAAAAGCGGCGTAGT GCTATCGGGTGAGCCCTGAGGTTTTCCCAGTCACGAC[c]
Km SceI P_{phoP} *adrA* Rv	TACCGCCTGTTCAACCGCTTTTCGGTAAAAATTTTCATCATTCA TTATTTTTGGGAACATTGCTTCCGGCTCGTATGTTG[c]
P_{phoP} *adrA* Fw	GTAGCAAGTTTATGAGCGCCTGCCTGAAAAAAGCGGCGTAGT GCTATCGGGTGAGCCCTGACTATTTGTCTGGTTTATTAACTG[d]
P_{phoP} adrA Rv	TACCGCCTGTTCAACCGCTTTTCGGTAAAAATTTTCATCATTCA TTATTTTTGGGAACATCTCGTCTCCCTTGTGTTAACAATAAGAA C[d]
01-E	CACAGTTGTTATAACGTTAC
***pgaABCD* expression from the *Salmonella* chromosome**	
SmaI *sb13* AB Fw	**CCCGGG**AACTGTATGTCATTGCCGTA
SpHI *sb13* AB Rv	**GCATGC**CCGATATAATCGAACGGCT
SpHI *sb13* CD Fw	**GCATGC**AGACGCCTGCTGATGAACT
SalI *sb13* CD Rv	**GTCGAC**TGCAGACGGAACTGGTTAA
BglII *sb13* AB Fw	**AGATCT**AACTGTATGTCATTGCCGTAC

BamHI *sb13* AB Rv	**GGATCC**ATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTC GTTTTACCGATATAATCGAACGGCTCAT
BamHI PcLrbs Fw	**GGATCC**AAATCTATCACCGCAAGGGA
sb13 PcL*pgaA* Rv	GCTACTTGAATACATGCGGTACCTTTCTCCTCTTT
PcL *pgaA* Fw	AAAGAGGAGAAAGGTACCGCATGTATTCAAGTAGC
PstI PcL *pgaA* Rv	**CTGCAG**CCCCGCCAGTTTATAGATAT
sb13 OK Fw	ATCGGTTGATTATGCCCGTCA
pgaA comp Rv	CCATTTGGTTTTCGGGCACC

pgaABCD operon expression in _E. coli_ under the PcL promoter

Km Pcl rbs *pga* Fw	CATTAGGAATAACAATTAAATCCGTGAGTGCCGTAGCGCAGCC TTTCATCAGGACTTTCGTTCGCTCAAGTTAGTAATTCTCAC[a]
Km Pcl rbs *pga* Rv	AGTAAGAAGTTTCAAAGCCCATTTGGTTTTCGGGCACCTTTTTC TGCTACTTGAATACATGCGGTACCTTTCTCCTCTTTAATG[b]

Mutation of the _pgaC_ gene in _E. coli_

pgaC Km SceI Fw	TAGACCTTATTCGTCCTGAGTTTTCAACAGCCTGGTATCCGAA AAATGATTAATCGCATCAGGTTTTCCCAGTCACGAC[c]
pgaC Km SceI Rv	TACCGGTTACGGCACCCACACGCGGGTTGTACAACATCGGTT CCACAATATATGCCGCCTGCTTCCGGCTCGTATGTTG[c]
Δ*pgaC*_Fw	TTTTCAACAGCCTGGTATCCGAAAAATGATTAATCGCATCGGC GGCATATATTGTGGAACCGATGTTGTACAACCCGCGT
Δ*pgaC*_Rv	ACGCGGGTTGTACAACATCGGTTCCACAATATATGCCGCCGAT GCGATTAATCATTTTTCGGATACCAGGCTGTTGAAAA

Construction of a collection of strains, derivative of ΔXII containing a single GGDEF domain protein

01-A	**GCGGCCGC**TGCCAGTGTAACTGTGGA[e]
01-D	**AGATCT**CTGGGACACGACCGTAA[e]
02-H	**GCGGCCGC**ATGAATTTGCATCATAAAGCG[e]
02-D	**AGATCT**GGCGATGCGCAGATAGT[e]
03-A	**GCGGCCGC**GATATCACCCAACAAATG[e]
03-D	**AGATCT**CAGATACGCCGGTAATTTT[e]
04-A	**GCGGCCGC**GGAATTGTCGTACACGGT[e]

04-D	**AGATCT**CTCACAACGAAATCCGCC[e]
05-A	**GCGGCCGC**ACCGGTAATTCAATCGCC[e]
05-D	**AGATCT**GTTTGAACAGGGCGTGC[e]
06-A2	**GCGGCCGC**CGTCATCCGTTCCTTGAACATAACGCGTCATCCG TTCCTTGAACATAACG[e]
06-D2	**AGATCT**CTGGGTTACATCTTCTACCCGGTCCTGGGTTACATCT TCTACCCGGTC[e]
07-A	**GCGGCCGC**GACGATATGGCAAAATAATG[e]
07-D	**AGATCT**AGCAACTTGAACAAGAGCA[e]
08-A	**GCGGCCGC**CACAGCATGGCGGTAAAA[e]
08-D	**AGATCT**GATATTGCCCGGCGTAC[e]
09-A	**GCGGCCGC**AGTTTCACCACAGGCGC[e]
09-D	**AGATCT**TTGAGAATAAAACGCAGTTG[e]
10-A	**GCGGCCGC**TATAGCCCGCAGGAATAC[e]
10-D	**AGATCT**ATCGAGCGTTGCCGGAT[e]
11-A	**GCGGCCGC**GTAAGATAACTGTGCGAAG[e]
11-D	**AGATCT**TCCTGATGCACATCAAGC[e]
12-A	**GCGGCCGC**TAACAGCTTAACGTTGTCC[e]
12-D	**AGATCT**CAGCTTGAAGCGTTGCTT[e]

Restriction enzymes recognition sites are shown in bold.

[a] Priming sequence for the Km resistance cassette underlined.

[b] Priming sequence for the PcL promoter underlined.

[c] Priming sequence for plasmid pWRG717 underlined, used for Km resistance cassette and I-SceI site amplification.

[d] Priming sequence for the *phoP* promoter underlined.

[e]Equivalences of gene names and the internal code used to name primers utilized for pKO3blue experiments: 01 (*adrA*); 02 (*yeaJ*); 03 (*yciR*); 04 (*sen1023*); 05 (*yegE*); 06 (*yfeA*); 07 (*yfiN*); 08 (*yhdA*); 09 (*sen3222*); 10 (*yhjK*); 11 (*sen4316*); 12 (*sen2484*).

RESULTS

Absence of the *pgaABCD* operon in *Salmonella* is likely the result of a secondary loss

To investigate whether the presence of the *pgaABCD* operon in *Escherichia* and its absence in *Salmonella* is due to a lineage-specific acquisition in *Escherichia* or to a loss in *Salmonella*, we performed different comparative and phylogenetic analyses (see Materials and Methods). Analysis of the genomic context of *E. coli* PgaA protein in the STRING database (Szklarczyk *et al.*, 2015) correctly identified the presence of four genes in the *pgaABCD* operon as significantly associated using exclusively gene neighborhood and gene co-occurrence information. The gene cluster, often only presenting the first three upstream genes, is widespread among *Enterobacteriaceae*, being present in 22 species of the 83 available in the database. Besides *Escherichia*, the genera with the cluster include *Klebsiella*, *Pectobacterium*, *Yersinia*, *Citrobacter*, and *Enterobacter*, among others. Analyses of the presence/absence of the genes revealed a similar pattern, confirming the absence of the genes in *Salmonella* species and other genera. Importantly, both analyses revealed a patchy presence/absence pattern, including many recent apparent losses within some genera such as *Citrobacter* or *Escherichia*. We then reconstructed individual phylogenies in each of the genes in the cluster by aligning the top 500 hits of a blastP search in NCBI nr database, after setting a filter to exclude sequences assigned to *E. coli*. All the top hits belonged to related species of *Enterobacteriaceae* excluding the possibility of recent, independent transfers of the cluster from a non-*Enterobacteriaceae* species. Maximum likelihood phylogenies of the four genes produced roughly similar topological arrangements of the included taxa (schematically depicted in Fig. 1A). We performed a similar analysis with *phoH*, the gene located in the vicinity of the cluster, encoding a protein with a nucleoside triphosphate

hydrolase domain. This gene has a broader distribution, present in 68 of the 83 taxa, a pattern suggesting a vertical inheritance with few independent losses. Importantly, however, for the shared species, the phylogenies of *phoH* and that of the four genes in the *pgaABCD* cluster showed an overall similarity (Fig. 1B). This indicates that the five genes followed a similar evolutionary history, with the exception of differential loss of genes in alternative lineages. This topology was congruent with the species tree for *Enterobacteriaceae* provided in the PATRIC database, which is based on the analysis of several shared genes (Williams *et al.*, 2010) (Wattam *et al.*, 2013), with the notable exception of the position of *Yersinia* or *Serratia* strains. Previous studies have shown that losses are more frequent than lateral transfer in the evolution of prokaryotic genomes (Snel *et al.*, 2002), and lateral transfer would generate discordance between gene trees (Soucy *et al.*, 2015). Hence, our results point to an overall dominance of vertical inheritance and differential gene loss in the evolution of this gene cluster within *Enterobacteriaceae*. Considering this scenario, the *pgaABCD* cluster was lost somewhere after the divergence of *Salmonella* and *Citrobacter* clades, and previous to the diversification of the currently sequenced *Salmonella* strains.

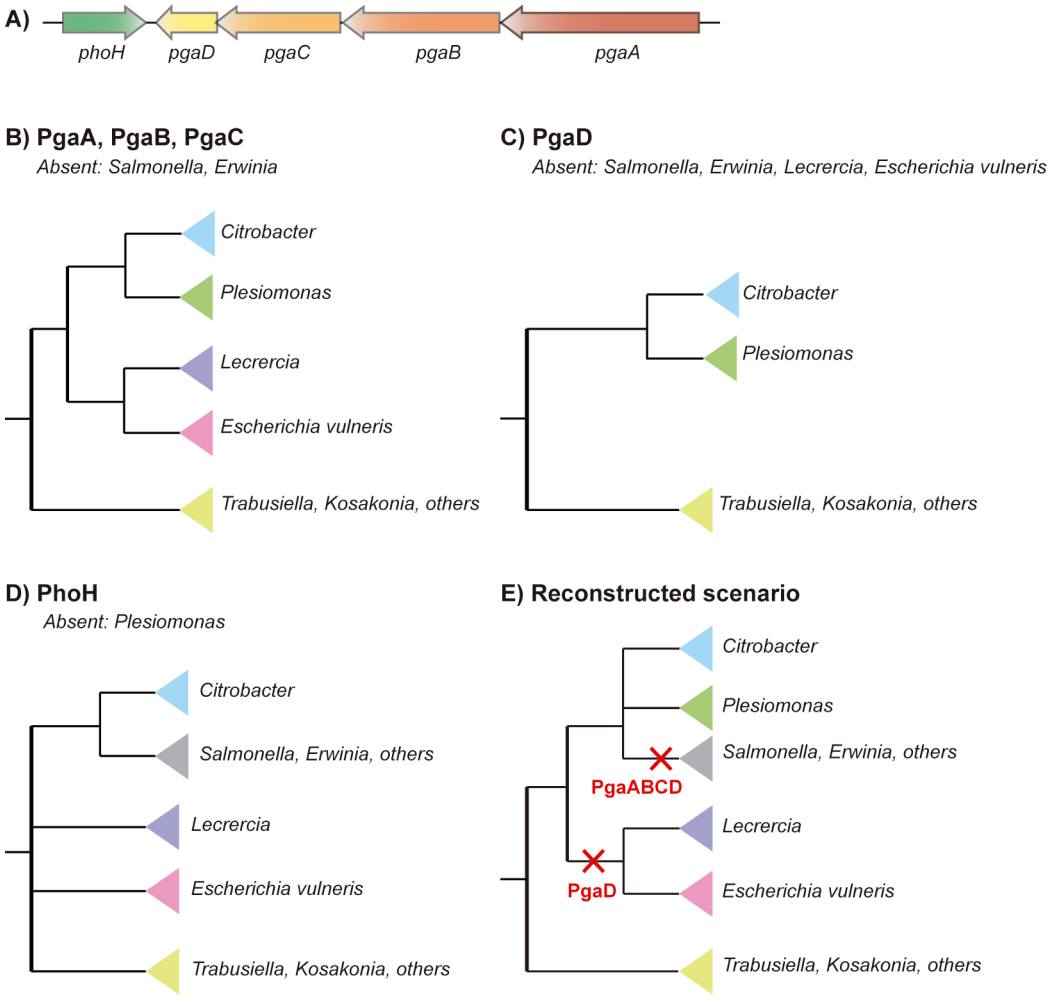

Fig. 1. Schematic representation of the phylogenetic relationships of homologues of the proteins analyzed in groups that are closely related to *Salmonella*. (A) Schematic representation of the *pgaABCD* operon and the *phoH* gene in *E. coli* K-12 MG1655. The proteins PgaA, PgaB, and PgaC showed the same pattern (B), with PgaD showing a more restricted distribution (C). PhoH showed a phylogenetic pattern (D) that is compatible with those of PgaABCD if three losses are inferred (E). PgaABCD in *Salmonella/Erwinia*, top red cross; PgaD in *Leclercia/E. Vulneris* (bottom red cross), and PhoH in *Plesiomonas* (not shown in the tree). The full phylogenies comprising the closest 250 homologues are provided in supplementary datafile 1

Expression of the *pgaABCD* operon in *Salmonella* is sufficient for PGA production

If *Salmonella* inability to synthesize PGA is exclusively due to loss of the *pgaABCD* operon, complementation with *pgaABCD* should be sufficient to restore PGA production. To test this hypothesis, we transformed a *S.* Enteritidis wild type strain with plasmid pJET::*pga* carrying the *pgaABCD* operon of *E. coli* MG1655 under the control of its own promoter, and analyzed PGA synthesis upon growth under *Salmonella* biofilm forming conditions (incubation in LB broth, at room temperature, without shaking) using a dot blot assay and an anti-PIA/PNAG antiserum. As expected, PGA was not detected in cell extracts of the wild type strain whereas WT pJET::*pga* produced PGA and accumulated it throughout the incubation time (Fig. 2A and Fig. S1A).

We next examined if, as it happens in *E. coli* (Steiner *et al.*, 2013), PGA production in *Salmonella* is also dependent on c-di-GMP. To do so, we firstly complemented *S.* Enteritidis ΔXII with the *pgaABCD* operon. *S.* Enteritidis ΔXII is a multiple mutant, derivative of the wild type strain, carrying mutations in all twelve genes encoding GGDEF domain proteins (putative c-di-GMP synthases) and thus incapable of synthesizing c-di-GMP (Solano *et al.*, 2009; Zorraquino *et al.*, 2013). The dot-blot assay showed that ΔXII pJET::*pga* was unable to produce PGA, confirming that c-di-GMP is indeed essential for PGA production in *Salmonella* (Fig. 2A). Secondly, we constructed a strain in which the *adrA* gene of *Salmonella*, which encodes a c-di-GMP synthase, is under the control of a constitutive promoter. This strain (WT PcL::adrA) constitutively produces high levels of c-di-GMP. Upon transformation with pJET::pga, this strain produced higher PGA levels than the wild type strain (Fig. 2A and Fig. S1A), showing that heterologous PGA synthesis in *Salmonella* is commensurate to cellular c-di-GMP levels. Finally, and in order to identify the source of c-di-GMP in WT

pJET::*pga* that triggers PGA production, we used a collection of twelve strains, derivatives of ΔXII, each of which contained the chromosomal copy of a single gene encoding a GGDEF domain protein in the original wild type genomic location (Solano *et al.*, 2009; Zorraquino *et al.*, 2013). The analysis of cell extracts of each strain complemented with pJET::*pga* showed that five GGDEF domain proteins, namely AdrA, YedQ, YegE, YfiN and SEN4316, when individually present in the chromosome of the cell, were able to elicit c-di-GMP dependent PGA synthesis (Fig. S1B). Overall, these results showed that heterologous *pgaABCD* expression is sufficient to restore *Salmonella* capacity to synthesize PGA and that this synthesis is dependent on c-di-GMP levels that are provided as a pool by different *Salmonella* c-di-GMP synthases.

Heterologous PGA production bestows biofilm lifestyle behavior upon *Salmonella*

In staphylococcal cells, production of PGA can be visualized as a ring of cells adhered to the glass wall at the air–liquid interface, when bacteria are incubated in a glass tube under shaking conditions (Valle *et al.*, 2003). To investigate whether *Salmonella* is likewise able to build a PGA mediated biofilm, we analyzed biofilm formation by WT pJET::*pga* and WT PcL::*adrA* pJET::*pga* after incubation in LB broth, at 28°C for 16 hours under shaking conditions. Only the second strain, which produces constitutive and high levels of c-di-GMP, produced a visible ring of bacteria adhered to the glass wall (Fig. 2B). Structure of this PGA based biofilm was then compared with the natural cellulose based biofilm formed by *Salmonella* using scanning electron microscopy (Fig. 2C). To do so, we used a cellulose overexpressing strain (WT PcL::*adrA*), a PGA positive and cellulose minus strain (*ΔbcsA* PcL::*adrA* pJET::*pga*) and a control strain that produces

neither cellulose nor PGA (*ΔbcsA* PcL::*adrA*). In the case of the PGA dependent biofilm, cells were tangled up in an abundant extracellular matrix mesh that interconnected the bacteria. Furthermore, spherical, knob-like structures were evident on the bacterial cell surface. These knob-like structures have already been described in PGA (PIA/PNAG) related biofilms of *E. coli*, *Yersinia pestis* and *Staphylococcus epidermidis* (Vuong, Voyich, *et al.*, 2004; Erickson *et al.*, 2008; Boehm *et al.*, 2009). On the other hand, bacteria inside a cellulose based biofilm were covered by a sheet-like material (Serra, Richter, and Hengge, 2013) that totally encased bacteria and that appeared more compact and structured than the PGA biofilm. To further investigate the differences between both types of biofilms, macrocolony biofilms were grown on LB agar plates (Fig. S2A) and a water-droplet analysis of colony hydrophobicity was performed (Cairns *et al.*, 2014). Results showed that a cellulose mediated biofilm is highly hydrophobic, whereas a PGA based biofilm exhibits intermediate hydrophobicity compared with the non-biofilm producing strain, *ΔbcsA* PcL::*adrA* (Fig. S2B). Collectively, these findings showed that heterologous PGA expression alongside high c-di-GMP levels enable *Salmonella* to build a PGA mediated biofilm that greatly differs at the structural level from the natural cellulose based biofilm.

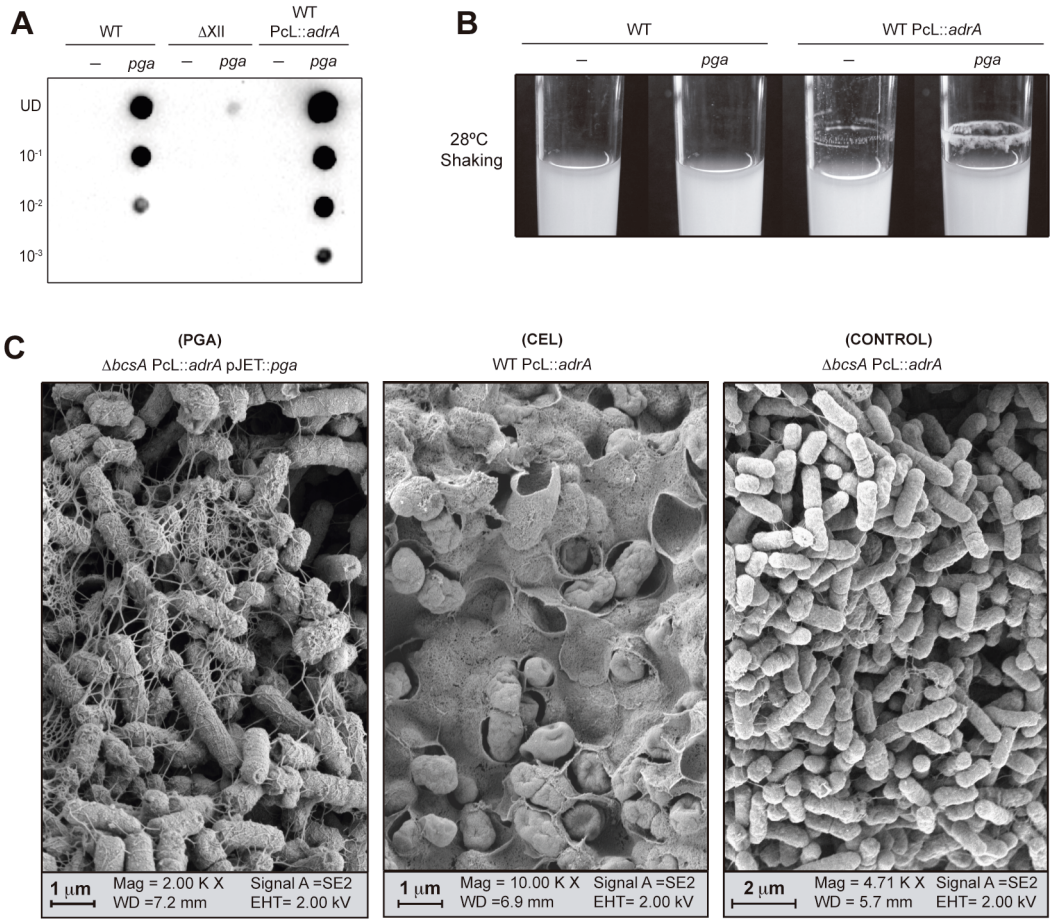

Fig. 2. Heterologous expression of the *pgaABCD* operon in *Salmonella* drives PGA synthesis in response to c-di-GMP levels and makes *Salmonella* able to build a PGA mediated biofilm. (A) Dot blot analysis of the PGA accumulated by *S.* Enteritidis wild type, ΔXII and WT PcL::*adrA* and their corresponding transformed strains with plasmid pJET::*pga* after 48 hours of incubation in LB or LB Cb broth, at room temperature, under static conditions. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PGA production was detected with specific anti PIA/PNAG antibodies. UD; undiluted sample. (B) Biofilm phenotypes of wild type, WT PcL::*adrA* and their corresponding transformed strains with plasmid pJET::*pga* after incubation in LB or LB Cb broth, at 28°C for 16 hours under shaking conditions. A ring of cells adhered to the glass wall at the air–liquid interface corresponds with a PGA based biofilm. (C) Scanning electron microscopy analysis of the biofilms developed by the PGA overproducing strain, *ΔbcsA* PcL::*adrA* pJET::*pga*, after incubation in LB broth, at 28°C for 16 hours under shaking conditions and by the cellulose overproducing strain, WT PcL::*adrA*, after incubation in LB broth, at room temperature under static conditions for 72 hours. A control strain, *ΔbcsA* PcL::*adrA*, that produces neither PGA nor cellulose was also analyzed after incubation in LB broth, at 28°C for 16 hours under shaking conditions.

Effects of PGA on *Salmonella* protection from environmental stresses

Biofilm exopolysaccharides provide protection from the external environment. Thus, a consequence of PGA loss might be a reduction in *Salmonella* resistance to environmental threats, unless another compound assumed such a function. To test this hypothesis, we compared the resistance provided by PGA and cellulose to several environmental stresses. Since it has already been described that cellulose mediates chlorine survival of *Salmonella* and other bacteria (Solano *et al.*, 2002; White *et al.*, 2006; Prigent-Combaret *et al.*, 2012), we first analyzed the susceptibility of macrocolony biofilms formed by the cellulose-positive strain (WT PcL::*adrA*) and the PGA-positive cellulose-negative strain (*ΔbcsA* PcL::*adrA* pJET::*pga*) to chlorine. The non-biofilm producing strain, *ΔbcsA* PcL::*adrA*, was used as a control. A 40 min exposure to sodium hypochlorite (200 p.p.m.) caused a decrease of ~5.5 logs in the number of control bacteria, compared to samples treated with only PBS (Fig. 3A). Conversely, the same sodium hypochlorite treatment caused a reduction of ~1 log in the number of bacteria inside a cellulose or a PGA based biofilm (Fig. 3A). These results determined that the protection against chlorine conferred by PGA is equivalent to that provided by cellulose.

Next, we tested the resistance that PGA and cellulose confer to five minutes of UV light irradiation. Although both exopolysaccharide overproducing strains survived better than the control strain that produces neither polysaccharide, the cellulose-positive cells showed a significantly higher survival rate than the PGA-positive cellulose-negative strain (Fig. 3B). Thus, under our experimental conditions, cellulose provides better protection against ultraviolet radiation than PGA.

Phages are found in abundance in environmental settings and

bacteria have developed sophisticated mechanisms, including biofilm formation, to limit phage reproduction. To address the impact of cellulose and PGA biofilm extracellular matrices on phage infection, we infected bacteria that had been grown on membrane filters under biofilm forming conditions with a P22 phage lysate and analyzed the transduction frequency of a streptomycin resistance cassette. Results showed that, under our experimental conditions, neither exopolysaccharide protected *Salmonella* from phage infection (Fig. 3C).

Overall, these findings suggested that PGA provides, at the most, similar benefits to those conferred by cellulose against environmental threats, at least under the conditions tested. Since both polysaccharides seem to have redundant roles in environmental survival, our results support the idea that during speciation the PGA pathway was lost without affecting survival outside the host during the *Salmonella* cyclic lifestyle.

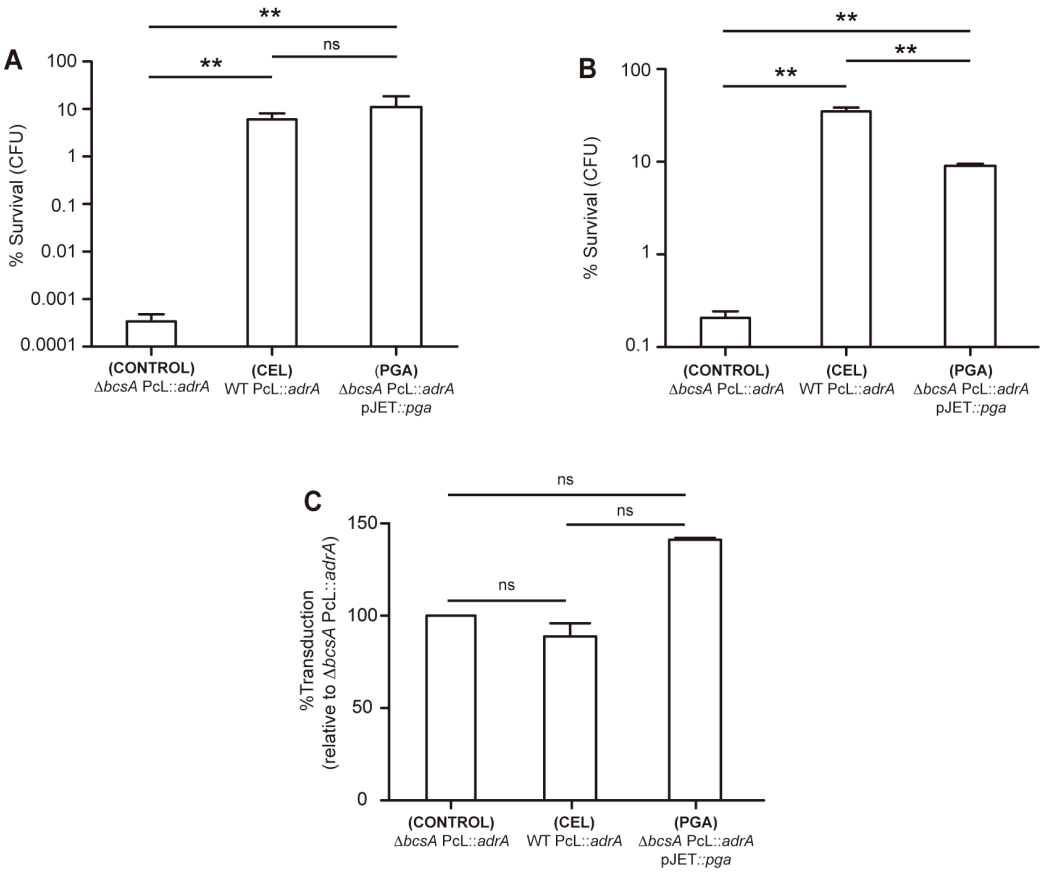

Fig. 3. PGA and cellulose confer equal protection to *Salmonella* from chlorine treatment, UV light irradiation and phage infection. (A) The cellulose overproducing strain, WT PcL::*adrA*, the PGA overproducing strain, *ΔbcsA* PcL::*adrA* pJET::*pga*, and the control strain, *ΔbcsA* PcL::*adrA*, were incubated on LB agar or LB agar Cb media for 48h at 28ºC. Macrocolonies were then exposed to 200 p.p.m. sodium hypochlorite for 40 min. Surviving bacteria were enumerated by viable plate counts, and their numbers were compared with that of control bacteria that had not been incubated with NaOCl. Note the logarithmic scale in the y axis. The data represent the mean of two independent experiments performed in triplicate. (B) Serial dilutions of the cellulose overproducing strain, the PGA overproducing strain and the exopolysaccharide minus strain were plated on N minimal medium containing carbenicillin and after 24 hours of incubation at 28°C, plates were exposed to five minutes of UV light irradiation. Replica plates were not subjected to treatment and served as controls. After 48 hours of incubation at 28°C, numbers of surviving bacteria were counted. Results are shown as % survival relative to untreated samples. *ΔbcsA* PcL::*adrA* and WT PcL::*adrA* carried an empty plasmid so that the three strains could be incubated on the same plates. Note the logarithmic scale in the y axis. The data represent the mean of three independent experiments. (C) Macrocolony biofilms were grown on polyvinylidene membranes and then subjected to phage infection with a P22 phage lysate generated from a streptomycin resistant strain. Transductants were enumerated by plate counts on LB Sm media and their numbers were compared with those of the exopolysaccharide minus strain, *ΔbcsA* PcL::*adrA*, which defined 100% transduction. The data represent the mean of three independent experiments. Statistical analysis in all assays was carried out using a Mann-Whitney *U* test. ns=no significant difference; * $P < 0.05$; ** $P < 0.01$.

PGA production hinders *Salmonella* intramacrophage survival

During infection, the ability of *Salmonella* to survive and replicate in the vacuole within host phagocytic cells is essential for systemic disease (Fields *et al.*, 1986). To investigate the consequences of PGA production in *Salmonella* intramacrophage replication, we tested the ability of a PGA producing strain to replicate in RAW264.7 murine macrophages and compared it with that of a cellulose producing strain. To guarantee the synthesis of PGA or cellulose inside macrophages, we created *Salmonella* strains displaying high c-di-GMP levels inside these cells through the use of the macrophage activated *phoP* promoter fused to the *adrA* gene (Pontes *et al.*, 2015). We firstly constructed WT P_{phoP}::*adrA* and confirmed that it produced a cellulose based biofilm in response to the low Mg^{2+} signal activating the *phoP* promoter (Fig. S3). Then, we engineered *ΔbcsA* P_{phoP}::*adrA* PcL::*pga*, a cellulose mutant that constitutively expresses the PGA synthesis machinery from the chromosome but that synthesizes PGA in a *phoP* dependent fashion (Fig. S3). As a control, we constructed WT *ΔbcsA* P_{phoP}::*adrA* producing neither cellulose nor PGA. The three strains were phagocytosed at similar rates and as it has already been described, the cellulose overproducing strain was defective for replication inside macrophages (Pontes *et al.*, 2015), showing an ~50% intramacrophage survival relative to the control strain *ΔbcsA* P_{phoP}::*adrA* (Fig. 4A). Remarkably, the PGA producing strain was significantly more attenuated than the cellulose overproducing strain, showing a 7% intramacrophage survival relative to the control strain (Fig. 4A).

Salmonella contained within the phagosomal environment encounter a diversity of antimicrobial factors including cationic antimicrobial peptides (CAMP) and reactive oxygen species (ROS) (Flannagan *et al.*, 2009). To investigate the cause(s) behind the low intramacrophage survival phenotype

related to PGA production, we firstly performed one-hour polymyxin susceptibility assays (Groisman *et al.*, 1997) of bacterial cells previously grown under low Mg^{2+} levels, a condition that promotes polymyxin resistance through activation of the PhoP regulon (Vescovi *et al.*, 1996; Groisman *et al.*, 1997). The presence of either polysaccharide, cellulose or PGA, did not have an effect on *Salmonella* polymyxin resistance (Fig. 4B). Then, we investigated whether reduced intracellular replication was linked to increased sensitivity to ROS production by assessing the ability to grow in the presence of 1mM H_2O_2 (Fig. 4C). When wild type *Salmonella* were inoculated into 1 mM peroxide-containing medium at 10^7 CFU/ml, there was no increase in cell numbers for the first 3 h of incubation, followed by fast recovery (Bogomolnaya *et al.*, 2013). Growth of the cellulose overproducing and control strains were indistinguishable from that of the wild type, whilst the PGA overproducing strain showed a significant viability loss throughout the incubation time (Fig. 4C). Taken together, these results indicated that PGA production has a detrimental effect on *Salmonella* intramacrophage survival and that such survival decrease may be partially explained by the fact that PGA makes *Salmonella* more sensitive to oxidative stress.

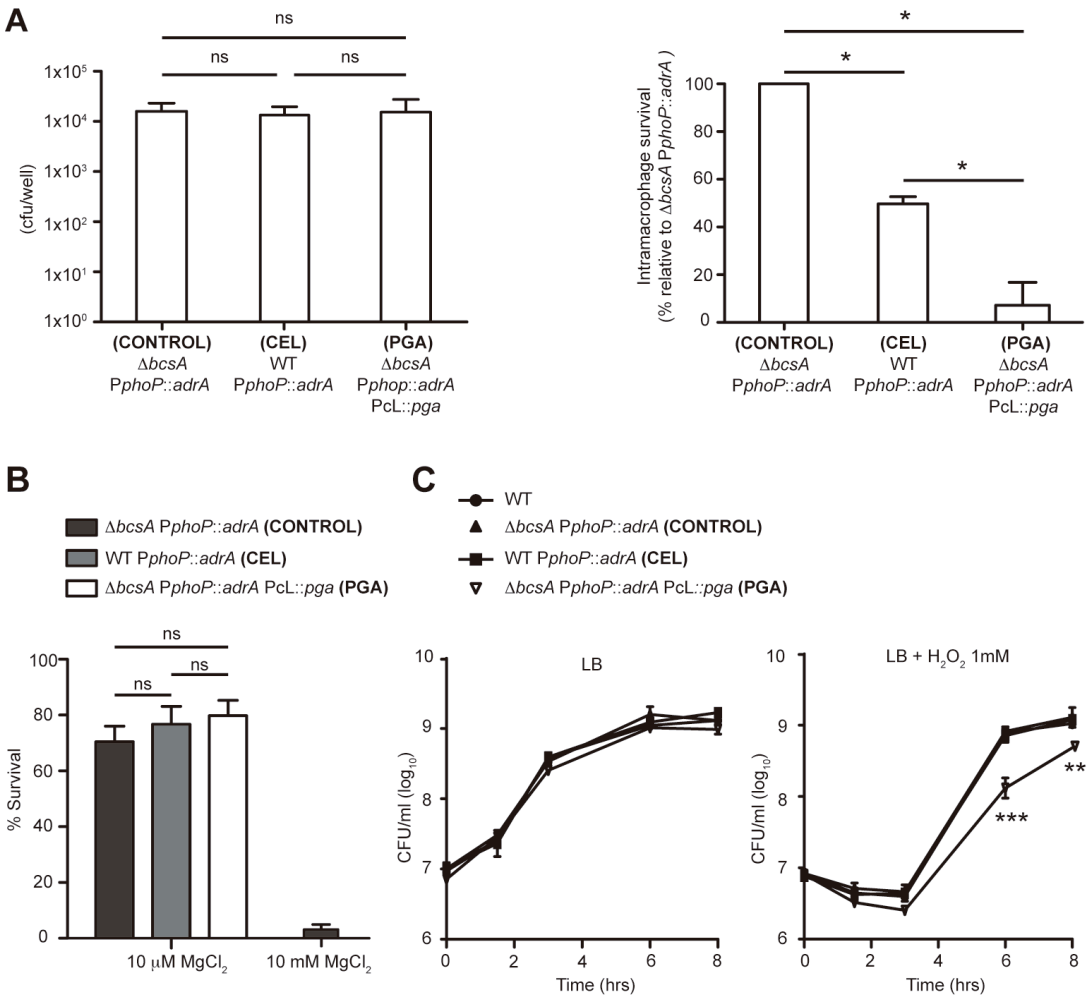

Fig. 4. A *Salmonella* strain that makes PGA inside macrophages is highly defective for intramacrophage survival and shows increased sensitivity to H_2O_2. (A) CFU of phagocytosed bacteria after a 1.5 h incubation with 2 × 10^5 RAW 264.7 macrophages at a multiplicity of infection of 10 (left panel). Replication within RAW 264.7 macrophages at 18 h postinfection (right panel). Replication of the exopolysaccharide minus strain, *ΔbcsA* P_{phoP}::*adrA*, defined 100% intramacrophage survival. Significance was determined by a Mann-Whitney *U* test. The data represent the mean of three independent experiments performed in triplicate. ns=no significant difference; * P < 0.05; ** P < 0.01. (B) Strains *ΔbcsA* P_{phoP}::*adrA*, WT P_{phoP}::*adrA* and *ΔbcsA* P_{phoP}::*adrA* PcL::*pga* were grown to logarithmic phase in N minimal medium with 10 μM $MgCl_2$. Washed bacteria were diluted 1:10 in LB and LB medium containing Polymyxin B at a final concentration of 2.5 μg ml^{-1}, and were incubated for 1 h at 37°C. Samples were diluted in PBS and plated on LB agar plates to assess bacterial viability. Survival values of bacteria incubated in LB Polymyxin are relative to values of bacteria incubated in LB. Strain *ΔbcsA* P_{phoP}::*adrA* incubated in N minimal medium with 10 mM $MgCl_2$ prior to polymyxin treatment showed a highly sensitive phenotype and served as a control of the resistance phenotype induced by low $MgCl_2$ concentrations. Data were analyzed by a Mann-Whitney *U* test. Bars are means of replicates ± s.e. (n = 3). ns=no significant difference. (C) Overnight cultures were diluted 1/100 in LB broth or LB broth supplemented with 1 mM H_2O_2 and incubated at 37°C with aeration. Aliquots were collected hourly, serially diluted and plated. Statistical analysis was carried out using a two-way analysis of variance combined with the Bonferroni test. ** P < 0.01; *** P < 0.001. Bars are means of replicates ± s.e. (n = 3).

PGA production renders *Salmonella* avirulent in mice

Since heterologous expression of PGA makes *Salmonella* less capable to survive inside macrophages, we hypothesized that PGA production might result in virulence attenuation upon infection by the natural oral route of BALB/c mice, which are susceptible to systemic infection with *Salmonella*. Taking into account that c-di-GMP is involved in modulating the innate immune response (Burdette *et al.*, 2011; Parvatiyar *et al.*, 2012), we constructed a *Salmonella* strain that constitutively produced PGA from the chromosome, without altering natural c-di-GMP levels. As expected, levels of PGA production by this strain, WT PcL*::pga,* were lower than those produced by WT pJET::*pga* (Fig. S4). Additionally, the *bcsA* gene was mutated in this strain, resulting in *ΔbcsA* PcL*::pga*, which produced PGA but not cellulose. Thus, virulence assays were carried out by comparing the pathogenic behavior of the control strain, *ΔbcsA*, which produces neither cellulose nor PGA, with that of either the PGA producing strain *ΔbcsA* PcL*::pga* or the wild type strain, which produces natural levels of cellulose during infection. These two strains did not show any discernable fitness cost compared to *ΔbcsA* when grown in LB broth at 37 °C (Fig. S5). Firstly, the impact of PGA and cellulose synthesis on the capacity of *Salmonella* to adhere and invade the intestinal epithelium was analyzed by carrying out a competitive index analysis in an ileal loop coinfection experiment (Fig. 5A). Both the wild type and *ΔbcsA* PcL*::pga* strains showed reduced capacity to adhere and invade the intestinal epithelium compared with the control strain, *ΔbcsA*. Secondly, we assessed the level of organ colonization following oral co-inoculation of the control strain, *ΔbcsA*, and either the wild type or *ΔbcsA* PcL*::pga* strain. In the case of mice co-infected with the wild type and *ΔbcsA* strains, the bacterial burden of the wild type was slightly higher than that of *ΔbcsA* in all organs analyzed (livers, spleens and gallbladders). Conversely,

the PGA producing strain showed to be extremely attenuated, since no *ΔbcsA* PcL*::pga* bacteria were recovered from the organs examined after co-infection with the control strain (Fig. 5B). To exclude the possibility that the control strain outcompetes the PGA producing strain when coinfection experiments are performed, we next compared the virulence of *ΔbcsA* and *ΔbcsA* PcL*::pga* strains by carrying out single infection experiments. Results confirmed that the PGA producing strain was highly attenuated, since mice inoculated with *ΔbcsA* PcL*::pga* did not show any disease symptom and most of them presented bacterial counts under the detection limit in livers, spleens and gallbladders (Fig. 5C). It is important to note that in the case of gallbladders, the entire organ was plated and that six out of seven gallbladders from mice inoculated with *ΔbcsA* PcL*::pga* were free from infection. Thus, these findings reflected the PGA impact on *Salmonella* intramacrophage survival and supported the view that heterologous PGA production impairs *Salmonella* survival in orally infected mice.

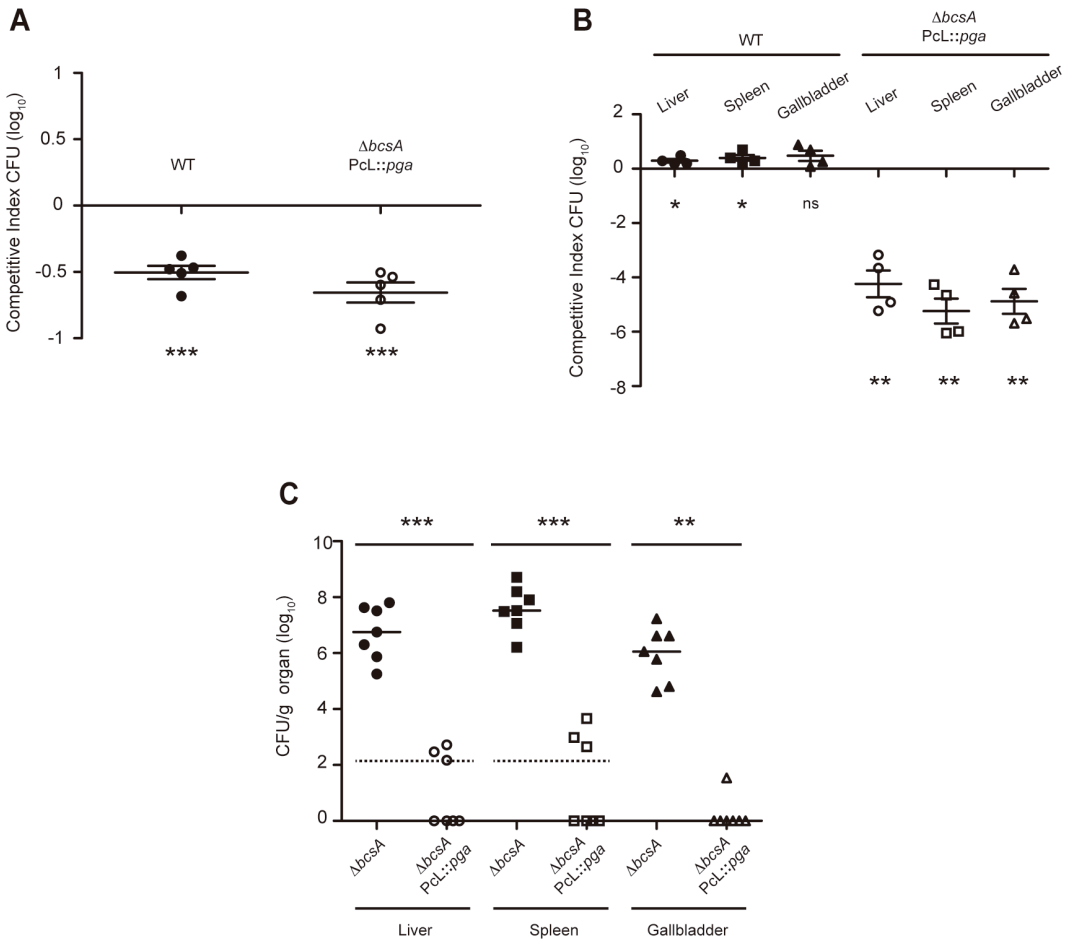

Fig. 5. PGA production leads to a complete *Salmonella* attenuation in a mouse model of infection. (A) Competitive index analysis of the wild type strain and *ΔbcsA* PcL::*pga* coinoculated with the control strain, *ΔbcsA*, after performing an ileal loop coinfection experiment. Five ileal loops were coinfected with $2x10^7$ cfu containing equal numbers of the control and either the cellulose or PGA producing strains. A CI > 0 indicates the exopolysaccharide producing strain with a colonization advantage compared to the control and a CI < 0 indicates the exopolysaccharide producing strain with a colonization disadvantage over the control. (B) CI analysis following intragastric inoculation of four BALBc mice with a 1:1 mixture of the control strain, *ΔbcsA*, and either the wild type or *ΔbcsA* PcL::*pga* strain (total inoculum administered was 2×10^8 cfu). Mice were sacrificed when evident signs of disease (score 2 to 3) were observed, and bacteria were enumerated from livers, spleens and gallbladders. No *ΔbcsA* PcL::*pga* bacteria were recovered from any of the organs analyzed; detection limit was 133 cfu/g in livers and spleens and 2 cfu/organ in the gallbladder. This detection limit was used to calculate the output corresponding to mice coinoculated with *ΔbcsA* and *ΔbcsA* PcL::*pga*. The plots display values obtained from individual samples and the mean CI is represented by horizontal bars. P-values were determined by a Student *t* test. ns = no significant difference; *P < 0.05; ** P < 0.01. (C) Virulence of *ΔbcsA* and *ΔbcsA* PcL::*pga* in mice inoculated intragastrically. Seven mice were infected with 1×10^8 cfu of the indicated strain. Mice inoculated with *ΔbcsA* were sacrificed when evident signs of disease (score 2 to 3) were observed, whilst mice inoculated with *ΔbcsA* PcL::*pga* were sacrificed at the end of the experiment. Bacterial loads in livers, spleens and gallbladders were analyzed. The dashed line indicates detection limit (133 cfu/g in livers and spleens). The plots display values obtained from individual samples and the median is represented by horizontal bars. Differences in colonization were statistically analyzed by using the Mann-Whitney U test. ** P < 0.01; *** P < 0.001.

PGA production makes *Salmonella* sensitive to bile salts

Bile resistance is indispensable for *Salmonella* to colonize the hepatobiliary tract during systemic infection and persist in the gall bladder during chronic infection (Andrews-Polymenis *et al.*, 2010; Gonzalez-Escobedo *et al.*, 2010) and again, this characteristic represents a major difference between *Enterobacteriaceae* species. Thus, to further examine the consequences of PGA production in the *Salmonella* infection process, we analyzed the ability of *Salmonella* PGA producing cells to cope with the presence of bile. Dilutions from cultures of the wild type, the PGA producing strain, *ΔbcsA* PcL::*pga*, and their corresponding exopolysaccharide minus strain, *ΔbcsA*, were spread on LB plates supplemented with 24% bile bovine. Exposure to bile caused a decrease of ~3 logs in the number of cfu of both the wild type and *ΔbcsA* strain, whereas it provoked a reduction of ~5 logs in the case of the PGA producing strain (Fig. 6A). Remarkably, PGA production in *E. coli* was also very detrimental for bile survival, since an *E. coli* strain producing PGA showed a ~2.5 logs reduction in bile sensitivity compared either with the wild type or with a *pgaC* mutant (Fig. 6B and Fig. S6). In order to analyze whether PGA production somehow alters membrane integrity, we tested the sensitivity of *Salmonella* and *E. coli* PGA producing strains to the ionic detergent SDS. The minimal inhibitory concentration (MIC) of SDS for the *Salmonella* wild type and *ΔbcsA* strains was 17%, whilst it decreased to 15% in the case of *ΔbcsA* PcL::*pga*. On the other hand, the MIC for *E. coli* MG1655 and *ΔpgaC* strains was found to be 15%, compared with 7% for the PGA producing strain MG1655 PcL::*pga*. Altogether, these results indicate that PGA causes a significant reduction in bile resistance both in *Salmonella* and *E. coli*, perhaps through modifications of membrane permeability.

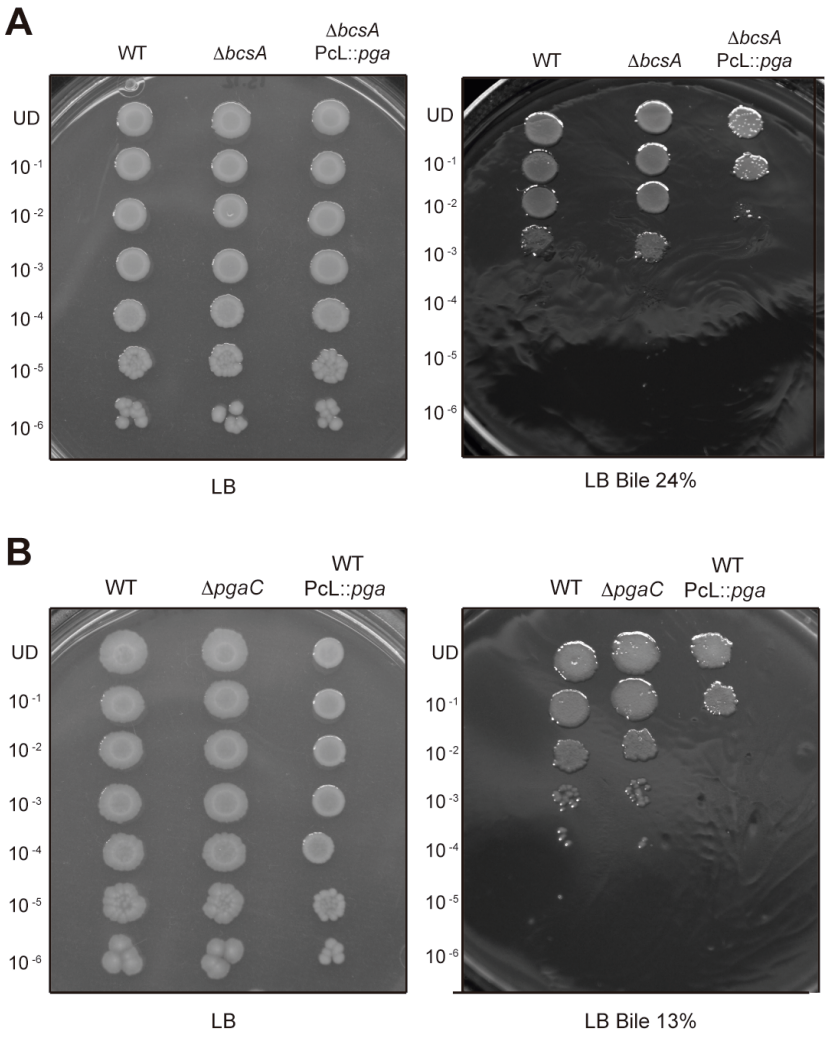

Fig. 6. Production of PGA in *Salmonella* and *E. coli* greatly increases bile sensitivity. (A) Bile sensitivity assay for the wild type, the PGA producing strain, *ΔbcsA* PcL::*pga*, and their corresponding exopolysaccharide minus strain, *ΔbcsA*. Two microliter portions of the appropriate dilutions of each culture were incubated for 24 h at 37°C in an LB Cb plate (left) or an LB Cb plate containing 24% bile bovine (right). (B) Bile sensitivity assay for *E. coli* MG1655, a MG1655 *pgaC* mutant and MG1655 PcL::*pga* strain that overproduces PGA. Two microliter portions of the appropriate dilutions of each culture were incubated for 24 h at 37°C in an LB plate (left) or an LB plate containing 13% bile bovine (right).

DISCUSSION

Acquisition of new genes is considered to be a mechanism to enhance an organism's ability to colonize a new environment, resist a specific antimicrobial or evade the immune system (Wiedenbeck and Cohan, 2011; Soucy *et al.*, 2015). However, genomic data reveal that gene loss is also a widespread strategy to enhance bacterial fitness (Maurelli *et al.*, 1998; Sokurenko *et al.*, 1999; Koskiniemi *et al.*, 2012; Hottes *et al.*, 2013; Bolotin and Hershberg, 2015; Albalat and Cañestro, 2016). There are at least two reasons why bacteria may loss genes during evolution. A gene product or pathway may become superfluous in the new environment. In the absence of purifying selection, the gene accumulates neutral mutations, generating pseudogenes that may be finally removed from the bacterial genome. Alternatively, the product of the gene may be detrimental, triggering selection to optimize bacterial fitness in the new environment. It is well established that *Salmonella* evolution towards virulence has, at least, involved the acquisition by horizontal gene transfer (HGT) of a virulence plasmid and several pathogenicity islands that contain the genes necessary for invasion of intestinal epithelial cells and the systemic phase of infection (Gulig *et al.*, 1993; Baumler *et al.*, 1998). However, the possibility that adaptation of *Salmonella* to the intracellular environment has occurred through gene loss has rarely been considered (Eswarappa *et al.*, 2009; Koskiniemi *et al.*, 2012). This work provides evidence that acquisition by *Salmonella* of an arsenal of virulence factors might have been useless in a strain producing PGA.

All bacterial species adapted to the mammalian intestine are resistant to the antibacterial activity of bile salts. However, the resistance of *Salmonella enterica* is especially remarkable. During systemic infection, *Salmonella* is able to transit from the liver into the gallbladder, where it can either induce inflammation and acute infection or persist chronically,

creating a carrier state (Crawford *et al.*, 2010; Bäumler *et al.*, 2011; Gonzalez-Escobedo and Gunn, 2013) . Several cell components and mechanisms have been related with *Salmonella* resistance to bile (Gunn, 2000; Sistrunk *et al.*, 2016). On one hand, different efflux pumps transport bile salts outside the cell decreasing their intracellular concentration (Piddock, 2006; Nikaido *et al.*, 2008). On the other, diverse strategies that involve membrane reorganization and provide barriers to reduce bile salts uptake have been described, such as remodeling the lipopolysaccharide (both lipid A and O-antigen), changing the length of the enterobacterial common antigen and reducing the content of the Braun lipoprotein bound to the peptidoglycan, the levels of muropeptides cross-linked by 3-3 peptide bridges and the amount of porins sensitive to bile (Van Velkinburgh and Gunn, 1999; Ramos-Morales *et al.*, 2003; Hernández *et al.*, 2012; Crawford *et al.*, 2012; May and Groisman, 2013; Hernández *et al.*, 2015). Our finding that constitutive production of PGA causes bile sensitivity in *S.* Enteritidis suggests an alternative strategy: the removal of compounds (PGA) that render the bacteria susceptible to bile. How PGA causes this effect is presently unclear. PGA represents an unusual bacterial exopolysaccharide, as some GlcNAc residues become deacetylated by the PgaB protein during secretion, providing a positive net charge to the polymer (Wang *et al.*, 2004; Itoh *et al.*, 2008). Thus, the presence of PGA may favor the accumulation of anionic bile salts on the bacterial surface and/or alter membrane permeability. The finding that PGA increased the susceptibility not only to bile bovine but also to the anionic detergent SDS support the hypothesis that PGA induces changes in membrane permeability.

We showed that constitutive expression of PGA also causes bile sensitivity in *E. coli*. These results raise the broader question of why *E. coli*, which displays a fair level of bile resistance necessary to grow in the small intestine, still produces PGA. Bile salts are maintained at high

concentrations in the duodenum, jejunum, and proximal ileum. In the distal ileum, bile salts are absorbed into the blood-stream, and the majority of bile is recycled back into the small intestine and does not enter the colon (Sistrunk *et al.*, 2016). *E. coli* resides in the microbiota found in the cecum and colon of humans. Thus, the presence of PGA might be compatible with the bile concentration in the small intestine and not with the concentration in the gallbladder. Alternatively, it is also possible that *E. coli* has developed regulatory systems to prevent PGA expression in the small intestine.

The second step of the infection process that is negatively affected by the presence of PGA is the survival and replication in the vacuole within host phagocytic cells. During systemic infection, *Salmonella* survives and replicates in vacuoles within host phagocytic cells where it must overcome the reactive oxygen species produced by macrophages (Fang, 2011). It has been reported that *Salmonella* needs to repress cellulose production inside the vacuole through the activation of MgtC, which prevents a rise in c-di-GMP (Pontes *et al.*, 2015). Increased levels of cellulose interfere with replication inside the vacuole and impair virulence in mice. The mechanisms underlying the antivirulence trait of cellulose has not been determined. We have now found that PGA production also hinders *Salmonella* division inside macrophages. Regarding this phenotype, we showed that production of PGA increases the susceptibility to H_2O_2 treatment, thus providing a potential mechanism for this attenuation. The notion that PGA is detrimental during infection of mammal cells is supported by studies with *Y. pestis* (Bobrov *et al.*, 2010). *Y. pestis* forms PGA mediated biofilms below 30ºC in the blood-feeding fleas favoring the transmission and invasiveness of the bacteria from fleas to mammals (Sun *et al.*, 2014). However, PGA production has to be inhibited in the mammal host over 30ºC to allow the development of a lethal infection. This temperature dependent regulation of PGA depends on the tight regulation of the c-di-GMP secondary

messenger.

Salmonella is an ubiquitous bacterium with a dual intracellular/extracellular lifestyle. Its extracellular life involves survival in the environment, a scenario in which exopolysaccharide-mediated biofilms play an important role, protecting bacteria against environmental threats. Our results indicate that PGA loss provides a fitness advantage when *Salmonella* colonizes the liver, gallbladder or resides inside the macrophages. However, loss of PGA might have negative consequences for survival in the environment unless another compound of the cell wall was able to compensate for PGA absence. Comparative phenotypic analysis between the protection conferred by PGA and cellulose against environmental threats revealed that PGA confers at the most similar benefits than cellulose, indicating that cellulose is sufficient to provide *Salmonella* with protection against environmental stresses and compensate for the loss of PGA function.

Our findings provide a plausible explanation for PGA loss from the *Salmonella* genome during evolution. They also enhance our understanding of the benefits and burdens of a widely used exopolysaccharide to form the bacterial biofilm matrix, highlighting the necessity of additional studies to depict the exact role of PGA at each step of the life cycle. Finally, our study may also encourage microbiologists to turn more attention towards gene loss research as an approach to obtain information about how pathogenic bacteria have adapted to the host.

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SUPPLEMENTARY MATERIAL

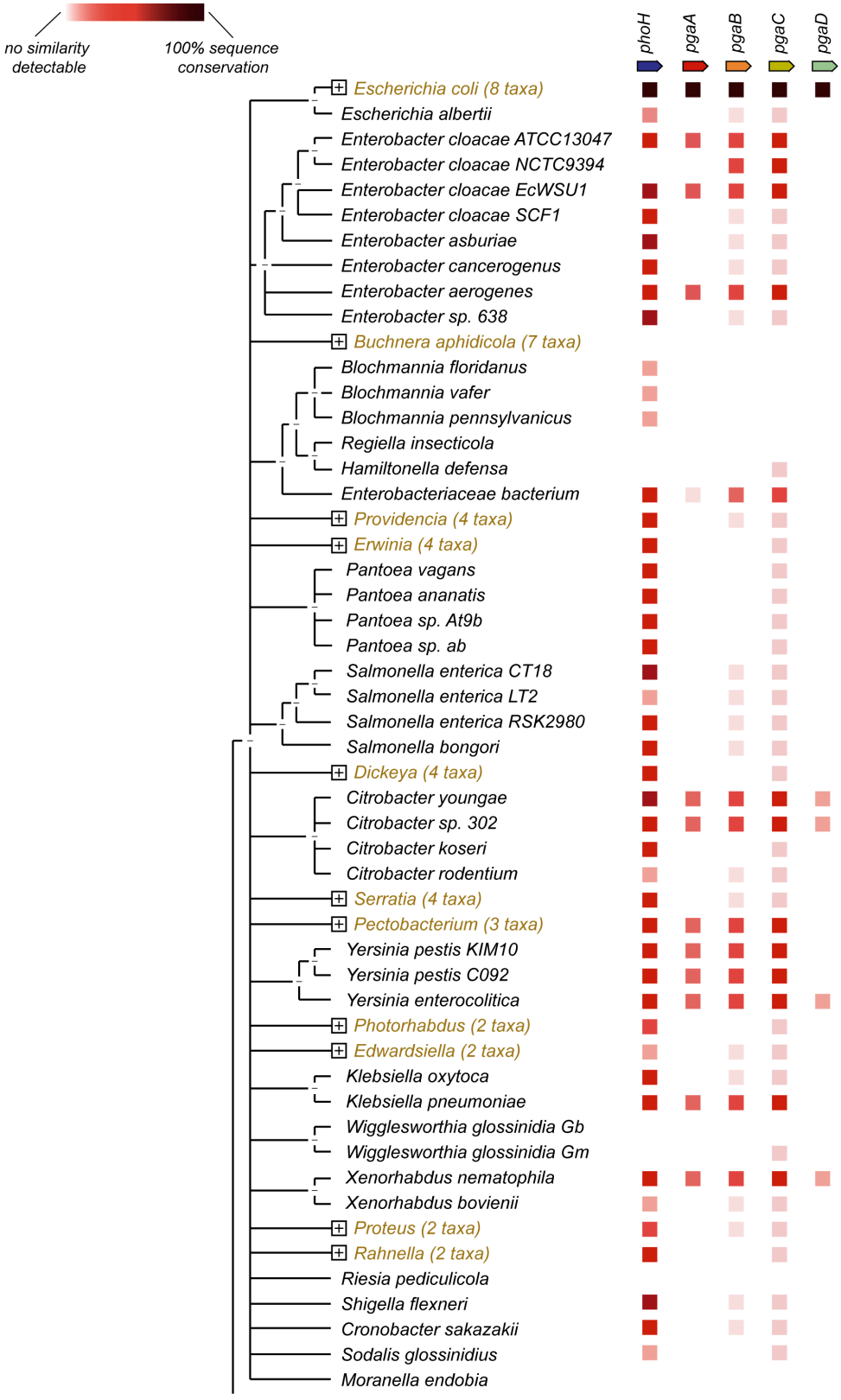

Fig. S1. Gene neighborhood analysis for *pgaA*, as provided in the STRING database (Szklarczyk *et al.,* 2015). Results for *Enterobacteriaceae* genomes are fully displayed, while those from other groups are presented in a collapsed mode.

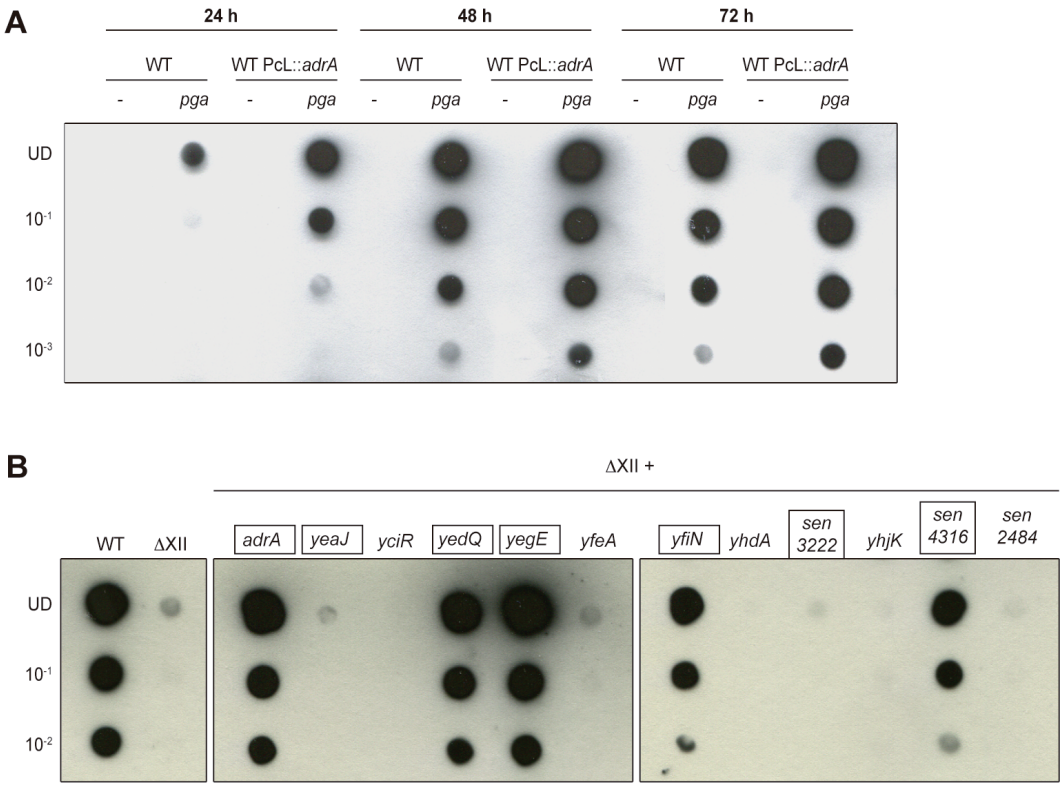

Fig. S2. Analysis of PGA synthesis dependence on c-di-GMP. Quantification of PGA exopolysaccharide production by dot blot. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PGA production was detected with specific anti PIA/PNAG antibodies. UD; undiluted sample. (A) Dot blot analysis of the PGA accumulated by *S.* Enteritidis wild type, WT PcL::***adrA*** and their corresponding transformed strains with plasmid pJET::***pga*** throughout 72 hours of incubation under *Salmonella* biofilm forming conditions, that is incubation in LB or LB Cb broth, at room temperature, under static conditions. (B) Dot blot analysis of the PGA accumulated by ΔXII and its twelve derivative strains, in which c-di-GMP synthesis is provided by a unique GGDEF domain protein. The wild type strain is shown as a control. All strains carried plasmid pJET::***pga*** and were grown for 72 hours in LB Cb broth, at room temperature, under static conditions. Seven genes that are shown in a black box encode predicted or demonstrated c-di-GMP synthases. Five of them, namely AdrA, YedQ, YegE, YfiN and SEN4316, were able to induce PGA synthesis. The rest of the proteins, namely YciR, YfeA, YhdA, YhjK and SEN2484, although harboring a GGDEF domain, are predicted or demonstrated phosphodiesterases (c-di-GMP degrading enzymes) or proteins with no c-di-GMP metabolic activity, and therefore, do not elicit PGA synthesis.

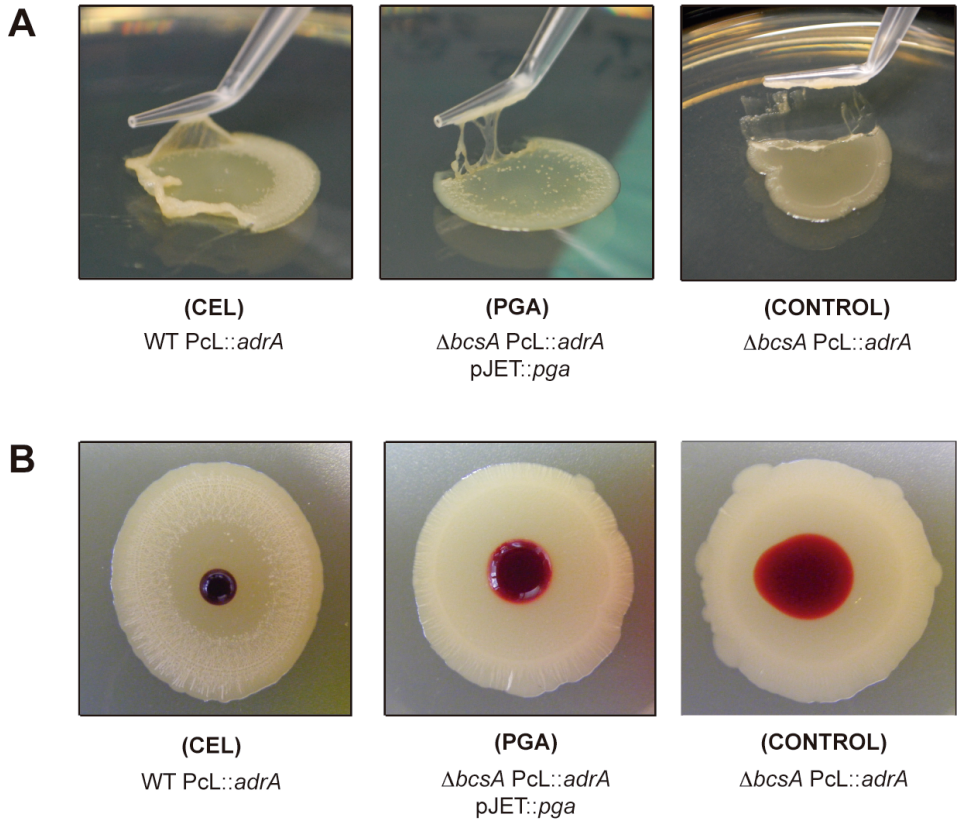

Fig. S3. Hydrophobicity analysis of macrocolony biofilms formed by WT PcL::*adrA* and *ΔbcsA* PcL::*adrA* pJET::*pga*. (A) The cellulose overproducing strain, WT PcL::*adrA*, the PGA overproducing strain, *ΔbcsA* PcL::*adrA* pJET::*pga* and the control strain, *ΔbcsA* PcL::*adrA*, were incubated on LB agar or LB agar Cb media for 48h at 28ºC. A bent tip was used to visualize the macrocolony appearance. The cellulose mediated macrocolony looked like a pellicle that was very difficult to disrupt whilst the PGA mediated macrocolony appeared much more mucoid. (B) Macrocolony biofilms were generated and a 10 µl water droplet stained with red food coloring was placed on each macrocolony, showing the hydrophobicity exhibited by the structure.

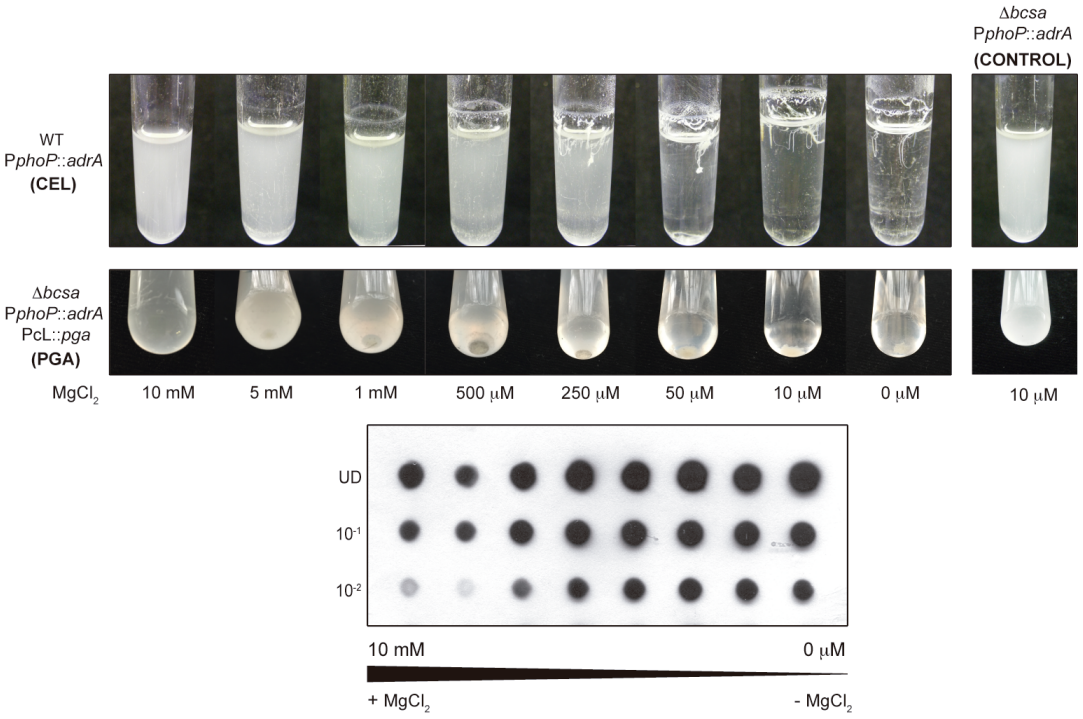

Fig. S4. Strains WT P_{phoP}::*adrA* and *ΔbcsA* P_{phoP}::*adrA* PcL::*pga* produce cellulose and PGA, respectively, in a *phoP* dependent fashion. A cellulose based biofilm is produced by WT P_{phoP}::*adrA* after overnight growth in low-Mg^{2+} liquid medium (top panel). An aggregate of *ΔbcsA* P_{phoP}::*adrA* PcL::*pga* bacteria can be observed at the bottom of the tube as Mg^{2+} concentrations decrease, indicative of PGA synthesis (low panel). Such aggregation correlates with increased PGA production detected by dot blot, using specific anti PIA/PNAG antibodies. Strain *ΔbcsA* P_{phoP}::*adrA* that produces neither cellulose nor PGA shows a planktonic phenotype.

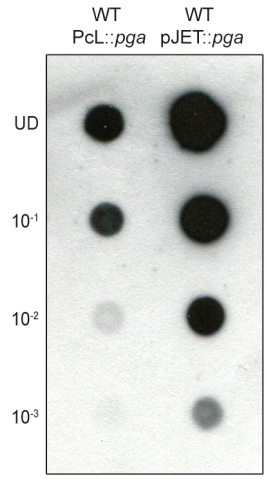

Fig. S5. Synthesis of PGA by a *Salmonella* strain that constitutively expresses the *pgaABCD* operon from the chromosome. Comparison of the PGA accumulated by WT PcL::*pga* and WT pJET::*pga*, expressing the *pgaABCD* operon from the chromosome or from a plasmid, respectively, after 24 hours of growth at 37°C in LB or LB Cb media. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PGA production was detected with specific anti PIA/PNAG antibodies. UD; undiluted sample.

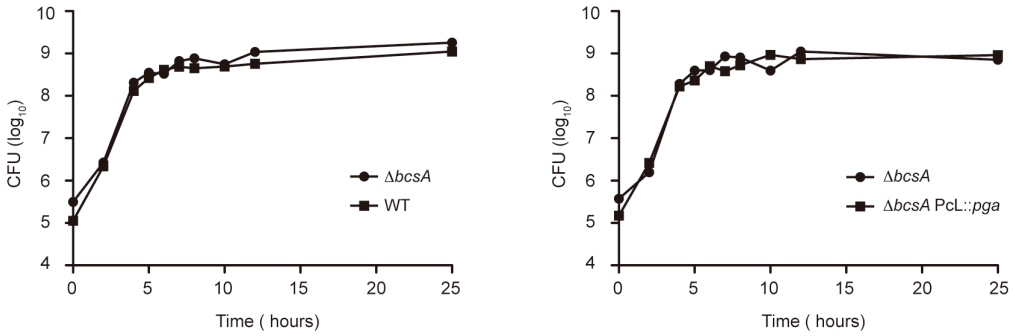

Fig. S6. Fitness of *ΔbcsA* versus the wild type or *ΔbcsA* PcL::*pga* strains. The competitive fitness of *ΔbcsA* in co-culture with either the wild type or *ΔbcsA* PcL::*pga* was determined by combining the two strains in LB broth, incubating at 37°C and enumerating each strain over time.

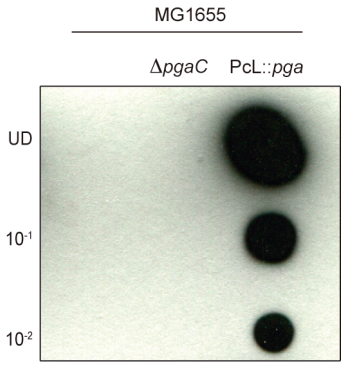

Fig. S7. PGA is overproduced by *E. coli* MG1655 when the *pgaABCD* operon is expressed from a constitutive promoter. Dot blot analysis of the PGA accumulated by *E. coli* MG1655, a *pgaC* mutant and a derivative of *E. coli* MG1655 in which the *pga*ABCD operon was placed under the control of the constitutive promoter PcL, after 24 hours of growth at 37°C in LB media. Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PGA production was detected with specific anti PIA/PNAG antibodies. UD; undiluted sample. Note that, as it has already been described (7), the parental *E. coli* MG1655 strain exhibited an extremely weak reaction with the antibody because of the posttransciptional repression of PGA production by the CsrA protein (8).

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FUTURE PROSPECTS

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Salmonella follows a cyclic lifestyle in which host colonization is followed by periods outside the host, where biofilm formation capacity plays a key role. Once inside the biofilm matrix, bacterial cells are up to 1000 times less susceptible to environmental stresses and disinfection treatments than planktonic cells (Costerton *et al.*, 1987). Protected by the biofilm matrix and probably also due to some physiological adaptations that occur during multicellular lifestyle, *Salmonella* biofilms in food processing plants, farms and fresh food constitute a source of contamination very difficult to eradicate. In this thesis, I have tried to expand our understanding of how biofilm related compounds have affected the evolution and pathogenicity of *Salmonella*, in the conviction that a deeper knowledge of the factors governing *Salmonella* multicellularity is essential to implement measures to control the infections caused by this pathogen. In this section, several questions raised by the results of this thesis as well as strategies that might be used to answer such questions are discussed.

1) Involvement of c-di-GMP in Salmonella virulence

The results obtained in the first chapter showed that c-di-GMP signaling is almost dispensable for *Salmonella* infection of BALB/c mice. The lack of c-di-GMP signaling only results in a slight but constant reduction of virulence in acute mice infection models. These results were somewhat unexpected because a signal transduction network that serves to connect environmental conditions with bacterial physiology was thought to be more relevant during adaptation to the conditions encountered inside the host. The finding that virulence capacity is slightly attenuated in the ΔXII mutant most likely reflects that a GGDEF domain containing protein is necessary during the infection process. To identify such protein, we are planning to perform virulence studies

with the collection of derivatives of ΔXII, each containing a single GGDEF-domain containing protein to evaluate if any diguanylate cyclase (DGC) is able to restore a virulent phenotype. Needless to say, this strategy would fail if more than one protein is necessary in the process. In the hypothetical case that a single DGC restored virulence, we would repeat similar virulence studies with a derivative strain producing a mutant version of the DGC without diguanylate cyclase activity. Intuitively, one would expect that the identified DGC affected virulence through its diguanylate cyclase activity. However, it cannot be excluded that DGCs might play additional roles independently of their capacity to produce c-di-GMP. Another strategy to evaluate whether c-di-GMP plays a role during infection would be to monitor c-di-GMP production inside the host. Recently, attempts to develop c-di-GMP reporters based on riboswitches (cis acting regulatory elements found in the 5' untranslated region (UTR) of some mRNAs) have been described (Bastet *et al.*, 2011). Riboswitches with the capacity to bind c-di-GMP have been described in *Bacillus spp., Vibrio cholerae* or *Clostridium difficile* species (Sudarsan *et al.*, 2008; Lee *et al.*, 2010; Bordeleau and Burrus, 2015). A reporter containing the c-di-GMP responsive riboswitch, under the control of a constitutive promoter and fused to the *luxABCDE* genes encoding luminescence proteins represents a conceivable tool for measuring c-di-GMP levels during mice infection (Hutchens and Luker, 2007). One possible limitation of this approach might be the affinity of the riboswitch for c-di-GMP. If the amount of c-di-GMP accumulated during infection is low, the sensitivity of the reporter might not be sufficient to detect its presence. Different strategies such as tandem fusion of riboswitches have been suggested to increase sensitivity (Zhou *et al.*, 2016).

A final aspect that is necessary to consider regarding c-di-GMP and *Salmonella* virulence is the role of c-di-GMP during chronic infections. Our results were obtained using acute mice infection models and therefore it cannot

be excluded that c-di-GMP may be important for establishing chronic infections where biofilm formation plays a key role. Thus, additional virulence studies using chronic models of infection are necessary to answer this question (Monack *et al.*, 2004).

2) ΔXIII strain as a carrier for the heterologous expression of antigens.

The results of chapter I established that the attenuated *Salmonella* ΔXIII strain is a safe and effective mean for inducing humoral and cellular immune responses in mice. Based on these two characteristics, attenuation and efficient activation of the immune response, strain ΔXIII appears to be a good vaccine candidate for the expression of heterologous antigens. The use of live attenuated *Salmonella* strains to deliver recombinant antigens to the immune system has been proposed for the development of multivalent vaccines (Hegazy and Hensel, 2012). Examples of heterologous antigens for expression in *Salmonella* ΔXIII, in order to develop a multivalent vaccine, are fimbrial adhesins and toxins from Enterotoxigenic *Escherichia coli* (ETEC). ETEC infections are the major cause of neonatal and post-weaning diarrhea in piglets, producing significant economic losses in pig farming and hundreds of millions of cases of diarrheal disease in humans every year. Another therapeutic use of *Salmonella* ΔXIII would be the treatment of solid tumors. The use of bacteria as cancer therapeutic agents has been followed for more than a century. Bacterial colonization of tumors may result in retardation of growth or even complete clearance of the tumor. In this regard, *Salmonella* has been intensively investigated for its potential to treat tumors (Cheng-Zhi Wang *et al.*, 2016) because it has the capacity to specifically migrate to the tumor region, infect tumoral cells and promote antitumor immunity as potent natural adjuvants, by activating innate immune cells followed by secretion of cytokines that can recruit and activate other immune cells at the tumor site (Henderson *et al.*,

1996). Although some *Salmonella* strains possess sufficient cytotoxicity against tumors, heterologous expression of toxins in *Salmonella* ΔXIII would allow the accumulation at high concentrations of chemotherapeutics, enhancing their antitumor effects.

One problem that we can envision for the use of *Salmonella* ΔXIII for heterologous expression of antigens is the unclear capacity of this strain for secreting proteins. We ignore how the simultaneous absence of c-di-GMP and RpoS might affect the functionality of the bacterial secretion machinery. Thus, it would be necessary to clarify this issue before considering the use of *Salmonella* ΔXIII for heterologous expression of antigens.

3) Specificity of c-di-GMP signaling

The presence of numerous proteins involved in c-di-GMP metabolism as well as c-di-GMP receptors in the same bacteria raises questions about the mechanisms that establish specificity of the c-di-GMP signaling pathways. In this respect, several possibilities have been proposed: i) colocalization of DGCs and their targets: c-di-GMP produced by a specific DGC affects the targets present in the near vicinity; ii) regulation of expression of c-di-GMP-related genes: there is a differential transcriptional regulation of c-di-GMP metabolizing enzymes for each particular environment or growth phase; and Iii) binding affinity of c-di-GMP receptors: specificity of c-di-GMP signaling is simply regulated through the binding affinity of c-di-GMP receptors. This last hypothesis implies the existence of a general pool of c-di-GMP whose level will depend on the number of GGDEF/EAL proteins present and active under each specific environmental condition. Using the collection of ΔXII derivatives, each of which contains the chromosomal copy of a single gene encoding a GGDEF domain protein, we have determined that five out of the eight DGCs from *Salmonella* have the ability to activate PGA synthesis. The finding that the PGA machinery is activated by c-di-GMP produced by many different DGCs,

including one that is unique to *Salmonella* (sen4316), disagrees with the hypothesis that regulation through c-di-GMP depends on the colocalization of the DGC with a receptor, in this particular case the PGA machinery. Furthermore, our results are in good agreement with previous studies showing that heterologous expression of HmsT, a diguanylate cyclase from *Yersinia pestis*, in ΔXII strain results in cellulose production (Solano *et al.*, 2009). Again, the localization of a DGC from *Yersinia* in the vicinity of the cellulose-synthase machinery in Salmonella, to locally activate cellulose production is counterintuitive.

Overall, these results support the hypothesis of the existence of a common pool of c-di-GMP that when reaching a higher level than the dissociation constant of PgaD, would have the capacity to activate PGA synthesis.

4) Involvement of bacterial polysaccharides in infection.

A striking characteristic shared by cellulose and PGA is that they are chosen by many phylogenetically unrelated bacteria, including gram negative and gram positive bacteria, to build the biofilm matrix. Furthermore, some bacterial species such as *Klebsiella pneumoniae*, *E. coli* or *Burkholderia* spp. produce both types of exopolysaccharides, indicating that there should not be any metabolic or functional incompatibility between both exopolysaccharides. Despite the fact that *E. coli* and *Salmonella* are closely related, *E. coli* maintains the capacity to produce both exopolysaccharides, whereas *Salmonella* only produces cellulose. In this study, we have identified some specific environmental niches (gallbladder and macrophages) where the presence of PGA is detrimental for *Salmonella* colonization. Based on these findings, we propose that *Salmonella* has favored the capacity to colonize these locations over the advantages that the presence of PGA might confer on bacteria. However, there are several questions related with the presence of these two exopolysaccharides that would require further investigation. For instance, are

both exopolysaccharides simultaneously present in *E. coli*? The finding that the synthesis of both exopolysaccharides is activated by c-di-GMP suggests that indeed, this should be the case. Otherwise, it would be difficult to understand how the synthesis of one exopolysaccharide might be inhibited when levels of c-di-GMP reach the dissociation constant of the receptor with lower affinity. We have tried to establish which of the exopolysaccharides is firstly synthesized in LB media, but unfortunately, the antibodies against cellulose were very inefficient at detecting cellulose in dot blot experiments and we could not obtain conclusive results. Alternatively, we are planning to use immunogold labeling and electron microscopy to determine whether both exopolysaccharides are simultaneously present on the bacterial surface. We know that the antibodies against PGA (kindly provided by G. Pier) are functional in this technique but we do not know whether the commercial antibodies against cellulose recognize cellulose under these conditions.

Another unsolved question relates with the properties that PGA confers to *E. coli* and whether these properties are different from the ones conferred by cellulose. Intuitively, the fact that several bacteria produce both exopolysaccharides strongly suggests that they are complementary and consequently that they do no confer similar properties. Alternatively, it is possible that both exopolysaccharides confer similar properties but due to their different biochemical nature, each one would assume its role under different environmental conditions. The phenotypic comparisons performed in this study have not unraveled any significant difference between cellulose and PGA but it would obviously be necessary to analyze other stresses and also, the same stress under different environmental conditions.

Finally, evidence is accumulating that production of exopolysaccharides are detrimental in some specific locations during infection of a mammalian host. Pontes et al (Pontes *et al.*, 2015) defined cellulose as an antivirulence factor, because stimulation of cellulose synthesis inside macrophages causes a

decrease in bacterial virulence. *Salmonella* solves the toxicity of cellulose in certain locations by tightly regulating cellulose levels through MgtC, a protein that inhibits the ATP synthase and consequently reduces ATP levels, preventing the rise of c-di-GMP. Similarly, the finding that PGA production is detrimental during *Salmonella* infection suggests that *E. coli* should also tightly regulate PGA production. In fact, *E. coli* regulates PGA production at a posttranscriptional level through CsrA, a small RNA binding protein that recognizes the leader sequence of the *pga* 5' UTR and blocks ribosome binding (Xin Wang *et al.*, 2005). In addition, PGA production is also regulated at a posttranslational level through the allosteric binding of c-di-GMP to PgaAD proteins (Steiner *et al.*, 2013). Consequently, loss of CsrA or increased levels of c-di-GMP augment PGA production. Therefore, PGA deregulation by any of these two approaches might be an interesting method to analyze PGA consequences during *E. coli* intestinal colonization. However, considering that CsrA and c-di-GMP regulate many other genes, the strategy of PGA derepression by modifying the levels of these regulators might probably be accompanied by many pleitropic effects. In this regard, it is very important to note that we have constructed a novel strain, MG1655 PcL::*pga*, described in Chapter III, in which the *pga* operon is expressed under the control of the PcL constitutive promoter and the consensus 5' UTR of λ T0 phage. Thus, in this strain, PGA expression is insensitive to CsrA activity and PGA production is perfectly detectable by Dot Blot. MG1655 PcL::*pga* can therefore be considered as a promising strain to evaluate the effects of PGA synthesis in *E. coli* host colonization.

Table 1: Comparative analysis of GGDEF domain containing proteins in *E.coli* and *Salmonella* genomes. The first column associated to each organism shows the genome position of all genes encoding a putative diguanylate cyclase protein (GGDEF domain containing protein). The second column shows the names of currently described genes. The aminoacid sequence of the catalytic A site of the GGDEF domain is then shown and conserved motifs are written in blue. Cells shaded in grey indicate an absence of the homologous protein in the corresponding organism. The last column associated to *Salmonella* shows the ability of each diguanylate cyclase to activate PGA production.

	***Salmonella* Enteritidis P125109**				***E. coli* K-12 MG1655**		
	Gene	**Name**	**Motif**	**PGA Synthesis**	**Gene**	**Name**	**motif**
GGDEF	*sen0368*	*adrA*	GGDEF	✓	*b0385*	*yaic*	GGDEF
	sen1769	*yeaJ*	GGDEF	X	*b1786*	*yeaJ*	GGDEF
	sen1023	*yedQ*	GGEEF	✓	*b1956*	*yedQ*	GGEEF
	sen2593	*yfiN*	GGDEF	✓	*b2604*	*yfiN*	GGDEF
	sen4316		GGEEF	✓			
					b1025	*ycdT*	GGEEF
					b1535	*ydeH*	GGDEF
					b1341	*ydaM*	GGDEF
					b1522	*yneF*	GGEEF
					b1785	*yeaI*	GEVFG
					b0834	*yliF*	GGDEF
					b1490	*dosC (yddV)*	GGDEF
					b1794	*yeaP*	GGDEF
GGDEF- EAL	*sen1329*	*yciR*	GGDEF-EAL	X	*b1285*	*gmR/yciR*	GGDEF-EAL
	sen2119	*yegE*	GGDEF-EAL	✓	*b2067*	*yegE*	GGDEF-EAL
	sen2396	*yfeA*	PGSEL-LEKE	X	*b2395*	*yfeA*	PGSEL
	sen3209	*yhdA*	HRSDF-ELM	X	*b3252*	*csrD (yhdA)*	HRSDF-ELM
	sen3222		GGDEF-EAL	X			
	sen3438	*yhjK*	SGYDF-EAL	X			
	sen2484		SGHDL-EAL	X			
					b1489	*dosP*	GGNGW-EAL

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CONCLUSIONS

CONCLUSIONS

1. Removal of all GGDEF domain containing proteins and thus c-di-GMP in *S.* Enteritidis causes a moderate loss of virulence in a murine model of infection, indicating that either a specific GGDEF-domain containing protein or c-di-GMP itself is required during infection.

2. A *S. Enteritidis* strain deficient in the c-di-GMP signaling network and *rpoS* (ΔXIII strain) is highly attenuated but capable of eliciting a cellular/humoral immune response against a lethal oral-challenge with a *S*. Typhimurium virulent strain.

3. The ability of S. Enteritidis ΔXIII strain to survive under desiccation conditions and to form a biofilm is seriously compromised, suggesting that its environmental survival and transmission to new hosts may probably be undermined. Also, ΔXIII shows DIVA features that allow differentiation of infected and vaccinated animals.

4. There is a Sting dependent IFN-β response in Salmonella infected macrophages, that is independent of the presence of c-di-GMP

5. STING deficient mice do not show any special susceptibility to a *Salmonella* infection, indicating that STING-mediated IFN-β does not play any detectable function during a *Salmonella* infection in mice.

6. Phylogenetic analyses suggest that the *pgaABCD* cluster was lost somewhere after the divergence of *Salmonella* and *Citrobacter*

clades, and previous to the diversification of the currently sequenced *Salmonella* strains

7. Heterologous *pgaABCD* expression is sufficient to restore *Salmonella* capacity to synthesize PGA in a c-di-GMP dependent manner. PGA production makes *Salmonella* able to a build biofilm that differs at a structural and functional point of view from the cellulose-mediated biofilm.

8. PGA production reduces *Salmonella* resistance against bile and its capacity to survive inside macrophages, rendering *Salmonella* avirulent in the mouse oral infection model.

9. PGA is an antivirulence factor whose loss may have been a necessary event during *Salmonella* speciation to permit survival inside the host.

CONCLUSIONES

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1. La mutación de todos los genes que codifican proteínas que contienen un dominio GGDEF y, por lo tanto, la ausencia de c-di-GMP en *S.* Enteritidis da lugar a una atenuación moderada de la virulencia en un modelo de infección murino, indicando que bien una proteína GGDEF específica o bien el propio c-di-GMP son necesarios durante la infección.

2. Una cepa de *S.* Enteritidis deficiente en la ruta de señalización del c-di-GMP y además mutante en *rpoS* (cepa ΔXIII) presenta una gran atenuación y es capaz de generar una respuesta inmune celular y humoral frente a un desafío, vía oral, con una cepa virulenta de *S.* Typhimurium.

3. La capacidad de la cepa *S.* Enteritidis ΔXIII de sobrevivir en condiciones de desecación y de formar un biofilm se encuentra seriamente comprometida, lo cual sugiere que su supervivencia en el ambiente y transmisión a nuevos huéspedes esté probablemente afectada. Además, la cepa ΔXIII posee características DIVA que podrían permitir la diferenciación de animales infectados y vacunados.

4. En macrófagos infectados con *Salmonella* se activa la producción de IFN-β dependiente de STING e independiente de la presencia de c-di-GMP.

5. Ratones deficientes en STING no muestran una susceptibilidad especial a la infección por *Salmonella*, lo cual indica que la producción de IFN-β mediada por STING no juega un papel significativo durante la infección por *Salmonella*.

6. Análisis filogenéticos sugieren que el operón *pgaABCD* se perdió en *Salmonella* después de la divergencia entre los géneros *Salmonella* y *Citrobacter* y previamente a la diversificación de las cepas de *Salmonella* que están actualmente secuenciadas.

7. La expresión heteróloga del operon *pgaABCD* es suficiente para restaurar la capacidad de *Salmonella* de sintetizar PGA, en una manera dependiente del c-di-GMP. La producción de PGA permite a *Salmonella* formar un biofilm que es diferente desde el punto de vista estructural y funcional al biofilm mediado por celulosa.

8. La producción de PGA disminuye la supervivencia de *Salmonella* tanto en presencia de sales biliares como en el interior del macrófago, lo cual da lugar a una atenuación total de la virulencia en un modelo de infección oral en ratón.

9. El PGA es un factor de antivirulencia cuya pérdida pudo haber supuesto un evento necesario durante la especiación de *Salmonella* para permitir su supervivencia en el interior del huésped.