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Analysis of the interaction between *Helicobacter pylori* and members of the Annexin family

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1 Introduction

1.1 Helicobacter pylori

1.1.1 Microbiology

Helicobacter pylori is a gram-negative, rod-like, spiral shaped bacterium (see Figure 1), which normally has 4-7 flagella at one pole, and is closely related to *Campylobacter jejuni*.[1,2] Apart from the usual spiral shape *H. pylori* can also appear in a coccoid form under certain circumstances.[3] This coccoid form is viable but non-culturable (VBNC) and is normally induced under stressful conditions.[3]

H. pylori is microaerophilic and is hence cultured in an atmosphere with reduced oxygen and elevated carbon dioxide.[1] It is both catalase- and oxidase-positive.[4]

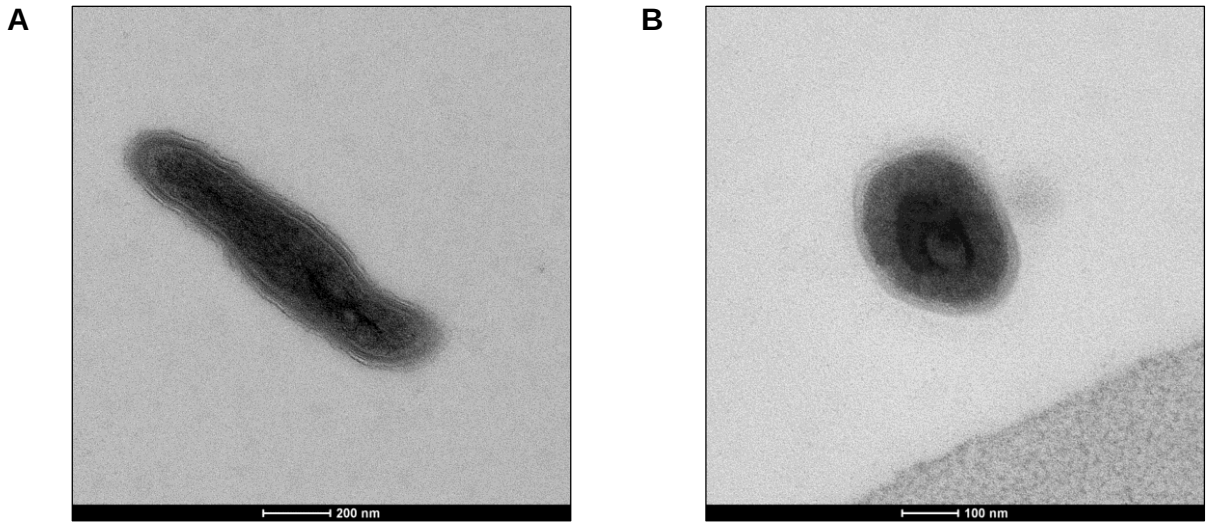


Figure 1 Electron microscopy of *Helicobacter pylori* wildtype strain P12

The P12 wildtype strain that was often used in this study was prepared for microscopy (see 2.2.1.6). Electron microscopy was then performed by Martin Sachse (Institut Pasteur, Paris, France).
A longitudinal (A) and cross section (B) are shown here. Note the helical form and the gram-negative typical double membrane of *H. pylori* in (A). Flagella were presumably lost during the preparation process.

Variations between different *H. pylori* strains, for example the expression of the Cytotoxin-associated gene A (*cag*) pathogenicity island (*cag*-PAI) and other pathogenicity factors, greatly influence their potential for persisting infection and disease development.[5-7]

The transmission route of *H. pylori* is most likely from person to person via an oral-to-oral or faecal-to-oral transfer and often occurs intrafamiliar.[8]

1.1.2 Epidemiology and discovery

H. pylori is prevalent all around the world and infects approximately 50 % of the human population. The prevalence of the infection in developing countries is notably higher than in developed countries.[9,10] Especially in developed countries the infection rate has been decreasing over the last years.[10-12] However, the *H. pylori* infection rate is not only dependent on the country of origin, but also positively correlated with the socioeconomic status during childhood.[10] In Germany the infection rate is low in small children (~3 %),[13] rises with increasing age and reaches nearly 50 % in adults.[8] *H. pylori* infection can cause various diseases (see 1.1.4) and leads to substantial consequences for both society and economy.

Though the first description of spiral bacteria in the gastric mucosa of patients was already given in 1889 by Jaworski, it took nearly 100 years before the findings of Barry Marshal and Robin Warren could change the generally accepted opinion of the scientific community that pathogens could not survive in the acidic stomach.[14] In the 1980s Barry Marshal and Robin Warren found the "unidentified curved bacilli"[1] in stomach biopsies from patients with gastric pathologies and were the first ones who were able to culture them.[1] In order to fulfil Koch´s third postulate (see 6.1) Barry Marshal drank a culture of *H. pylori* and consequently developed gastrointestinal symptoms as well as acute gastritis.[15] In 2005 the two scientists were awarded the Nobel Prize in Physiology or Medicine.

1.1.3 Pathogenic mechanisms

H. pylori is a human pathogen that colonizes the stomach mucosa. The human stomach is a hostile environment for bacteria due to its highly acidic pH, its mucus layer acting as physical impediment and its potent immune system.[16] Nevertheless, *H. pylori* can successfully colonize and persist in this environment because of its many pathogenicity factors (see Figure 2 and text below for details of the pathogenic mechanisms in an *H. pylori* infection).

The urease enzyme is a major pathogenicity factor of *H. pylori* and accounts for approximately 10 % of the total cellular protein.[17] It has been shown to be essential for the survival and colonization of the bacteria in the host´s stomach.[18] The urease of *H. pylori* is mainly expressed in the cytoplasm of the bacteria, but it has been published that bacteria can bind and take up the protein after the lysis of other bacteria, which is then surface-associated.[19] The urease enzyme catalyses a reaction of urea and water to ammonia and carbon dioxide, therefore adjusts the periplasmic pH and nearly neutralizes the acidic micro-environment of the bacteria.[20,21] However, the urease does not only play a key role in the elevation of the pH in the stomach lumen, but also in the mucus layer. The rise in the pH changes the rheological behaviour of the mucus. This change, together with the helical cell

shape and the flagella movement of the bacteria, enables *H. pylori* to move in the now viscous liquid mucus (see Figure 2).[22-24]

Another relevant part of the pathogenicity of *H. pylori* is its adhesion to the gastric epithelial cells. There are several adhesins like blood group antigen-binding adhesin A (BabA), sialic acid-binding adhesin (SabA), adherence-associated proteins (AlpA, AlpB), and other members of the *H. pylori* outer membrane protein families (Hop and Hor).[20] Some are known to interact with receptors on the host cell surface and allow the bacterium to colonize and persist in its host as well as release its toxins into the host cell.[20,25]

After adhesion *H. pylori* can inject one of its major pathogenicity factors, the Cytotoxin-associated gene A (CagA), in the host cell via the type IV secretion system (T4SS), which is encoded on the *cag*-PAI.[26,27] After injection, CagA is phosphorylated on tyrosine residues and causes various effects in the host cell (see Figure 2).[28] Upon transmission and afterwards CagA interacts with the phospholipid phosphatidylserine (PS) in the inner leaflet of the host´s plasma membrane.[29] CagA induces diverse changes in the host cell, both phosphorylation-dependently and phosphorylation-independently: the polarity of the epithelial cells is affected, the cell-cell junctions are disrupted and the cells consequently lose adhesion.[30] Furthermore, CagA and other components of the T4SS can induce an interleukin 8 (IL-8) secretion of the host cell, though the exact mechanisms are still controversially discussed and remain partly unknown.[6,31-33] The phosphorylated CagA can also interfere in the intracellular signalling of the host cell by interacting with host proteins containing so called Src homology 2-domains (SHP-2).[34,35]

Another important pathogenicity factor of *H. pylori*, the Vacuolating cytotoxin A (VacA) is secreted through a type V secretion system (T5SS), but is not directly injected into the host cell. While the majority is in this free-soluble form, about one quarter of VacA is released while associated to outer membrane vesicles (OMVs).[36] After binding to the host cell VacA induces the formation of vacuoles, leading to cell damage (see Figure 2).[37] But the vacuolation is only one of many effects VacA generates. It can: induce autophagy and cell death of both epithelial and immune cells, be responsible for changes in the mitochondria, interfere with cell signalling and cause increased membrane permeability.[38]

Additionally, *H. pylori* has developed several strategies to evade the human immune system. They are crucial for the infection and persistence of the pathogen in the stomach.[16] Amongst these are various modifications of its lipopolysaccharide (LPS), which shows a low endo-toxicity compared to other bacteria and mimics human blood group antigens (see 1.1.6).[39,40]

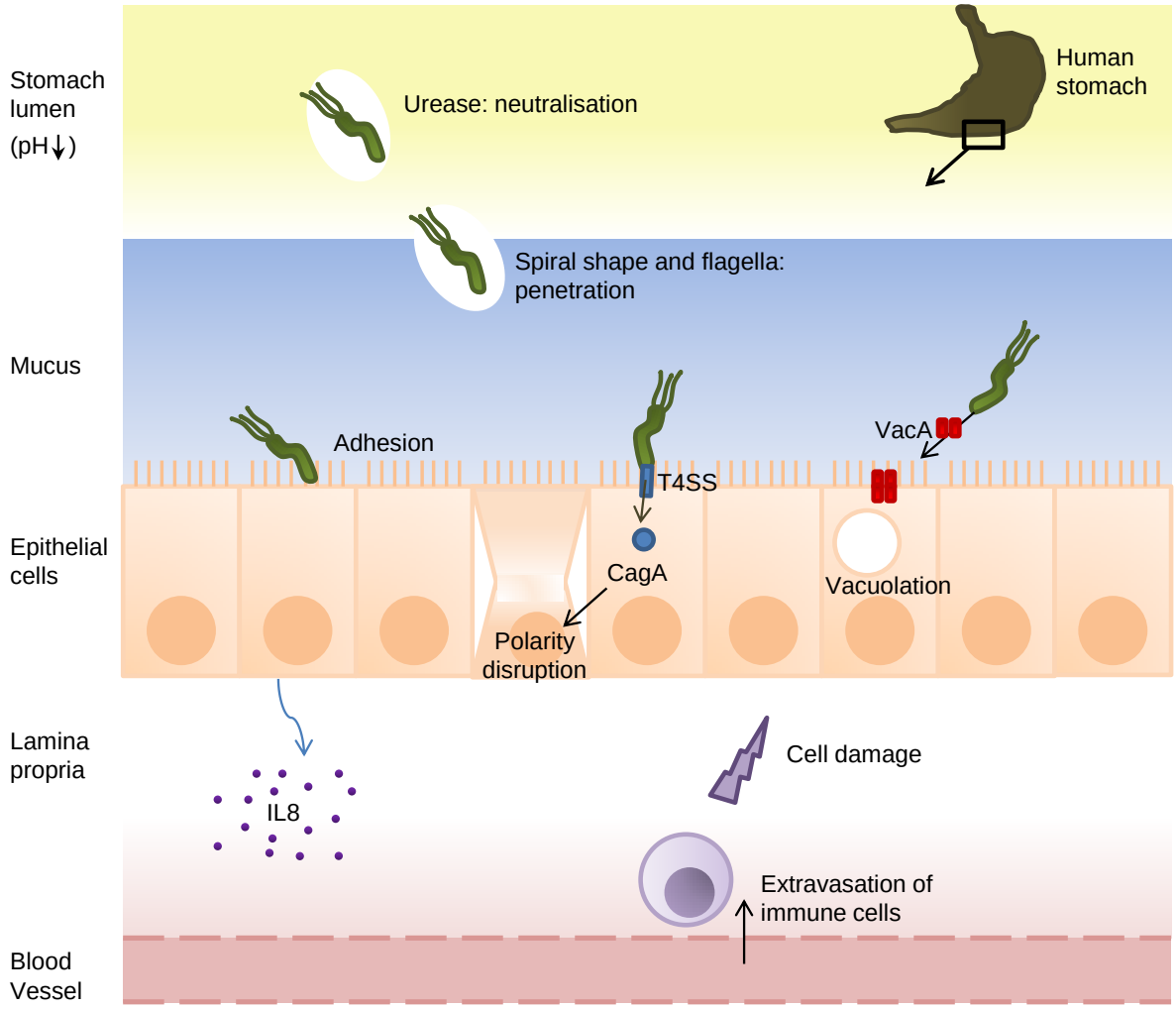


Figure 2 Pathophysiology of *Helicobacter pylori* infection

Various virulence factors enable *H. pylori* to infect and persist in the human stomach.
The enzyme urease neutralizes the acidic environment in the stomach lumen and liquefies the gastric mucus. *H. pylori´s* spiral shape and flagella allow the penetration of the mucus. Adhesion is essential for the effect of the pathogenic factor Cytotoxin-associated gene A (CagA), which is injected in the host cell via a type IV secretion system (T4SS). The Vacuolating cytotoxin A (VacA) is secreted and leads i.a. to vacuolation.
As a response to the infection with *H. pylori* the interleukin 8 (IL-8) secretion is enhanced and immune cells extravasate from the blood vessels, creating inflammation and causing cell damage.
Figure adapted and modified from Suerbaum S. et al. [41]; p 284.

1.1.4 Pathophysiology and clinical effects

An infection with *H. pylori* is strongly associated with a variety of gastric pathologies. Approximately 15 % of patients with an *H. pylori* infection will develop a peptic ulcer disease, while nearly all of them show histological features of chronic active gastritis.[9,42,43] The infection predominantly occurs in the antrum part of the stomach.[44] *H. pylori* is also classified as group 1 carcinogen and an infection with the pathogen is a major cause for the development of gastric adenocarcinoma.[45,46] 89 % of non-cardia gastric cancer and 17.8 % of gastric cardia carcinoma can be attributed to *H. pylori*.[47] There were 770,000 cases of

H. pylori-associated cancer in the world in 2012 and the bacterium can be seen as one of the most important infecting agents causing cancer, next to human papillomavirus and hepatitis B and C virus.[47,48] Not only adenocarcinoma but also gastric mucosa-associated lymphoid tissue lymphoma (MALT lymphoma) is highly associated with an *H. pylori* infection.[49] It is important to point out that not only the bacterium itself, but mainly the response of the host´s immune system is responsible for the induced cell damage. The clinical progression and outcome of an *H. pylori* infection depends on the combination of bacterial, host and environmental factors.[9]

Surprisingly an infection with *H. pylori* is inversely associated with some other pathologies. The risk to get oesophageal cancer is reduced when an *H. pylori* infection is present.[50] Though the exact underlying mechanisms remain unclear, epidemiological data suggest that the prevalence of atopies (such as allergies and asthma) as well as inflammatory bowel disease is lower in patients with an *H. pylori* infection than in non-infected individuals.[51,52]

1.1.5 Diagnostics and treatment

One of the gold standards for the diagnosis of an *H. pylori* infection is gastro-oesophageal endoscopy with following histology of the biopsy samples.[53] To ensure a reliable diagnosis the Sydney System guidelines were created.[54] These guidelines state that biopsies should be taken from both the antrum and the corpus of the stomach and that at least two samples should be taken from each region.[55]

A variety of further invasive and non-invasive tests can be used to detect an *H. pylori* infection (see Table 1) and the suitable method should be selected based on the clinical circumstances of each patient as well as the availability and practicability of the respective method.[8,53,56]

An *H. pylori* eradication therapy is highly indicated when patients suffer from peptic ulcer disease, gastric MALT lymphoma, or functional dyspepsia with an *H. pylori* infection.[8,57-59]

For a long time, the triple therapy with proton pump inhibitor (PPI), Clarithromycin and either Amoxicillin or Metronidazole has been considered the standard first line treatment.[60] But especially in settings with a high Clarithromycin resistance a Bismuth quadruple therapy (PPI, Bismuth salts, Tetracycline and Metronidazole) should be used as alternative first line treatment. A non-Bismuth quadruple therapy (PPI, Clarithromycin, Amoxicillin and Metronidazole) can be used alternatively, if Bismuth is not available.[61]

Table 1 Different diagnostic tools

The table shows the different diagnostic options recommended by the German "S2k-guideline *Helicobacter pylori* and gastroduodenal ulcer disease" and the corresponding sensitivity and specificity.
Table was adapted from the above mentioned guidelines.[8]

		Sensitivity [%]	**Specificity** [%]
Invasive methods	*Culture*	70-90	100
	Histology	80-98	90-98
	Rapid urease test	90-95	90-95
	PCR	90-95	90-95
Non-invasive methods	*Urea breath test*	85-95	85-95
	Stool antigen test	85-95	85-95
	Serology antibody test	70-90	70-90

As a consequence of the increasing development of antibiotic resistance and because of the large number of asymptomatic *H. pylori* infections, there has been a constant striving for the development of a vaccine against *H. pylori* to prevent gastric adenocarcinoma.[62-64] A Chinese research team published a phase three trial in 2015, successfully using an oral recombinant vaccine in children.[65] However, a critical analysis and further work is needed.[66,67]

1.1.6 *Helicobacter pylori* and lipids

The cell envelope of most gram-negative bacteria consists of various layers: the cytoplasmic membrane, peptidoglycans, the periplasmic space and the outer membrane (see Figure 3A). While the inner, cytoplasmic membrane is quite similar to other biological membranes, the presence of LPS makes the outer membrane of gram-negative bacteria quite unique.[68] LPS consists of lipid A, which is the connection to the membrane, a core oligosaccharide and an O side chain (see Figure 3B). LPS can strongly activate the host´s innate immune system, serving "as a molecular signal"[39] for danger and being recognized by the host´s cationic antimicrobial peptides (CAMPs).[39]

The cell envelope of *H. pylori* is comparable to that of other gram-negative bacteria, though there are some specifics (as outlined below).[69]

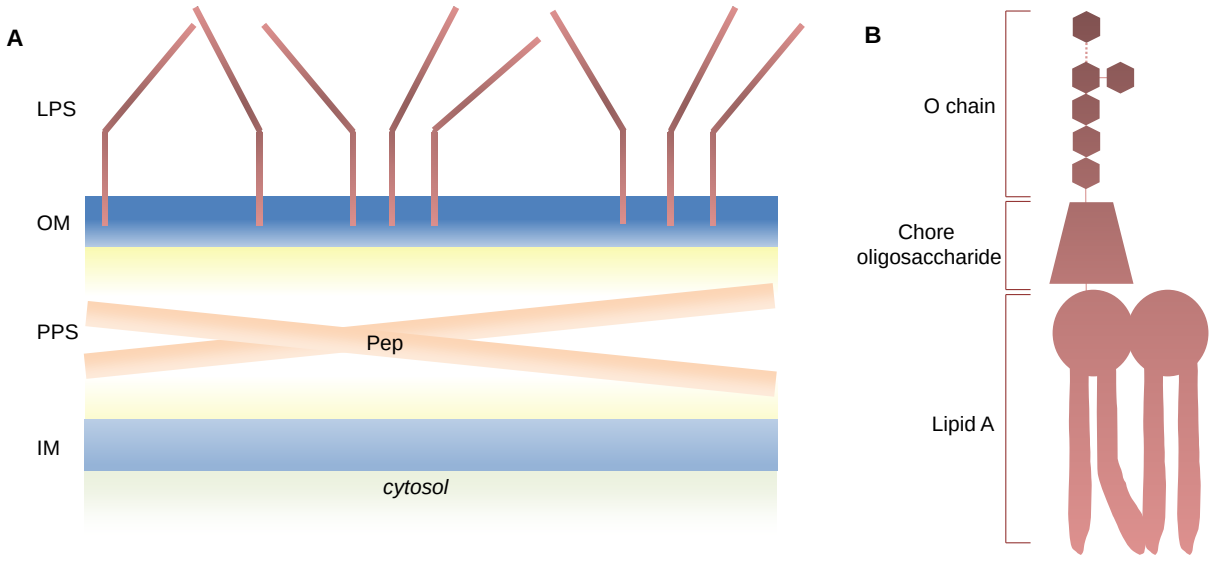


Figure 3 Gram-negative cell membrane and structure of LPS

(A) Model of the layers of the membrane of gram-negative bacteria.
Lipopolysaccharide (LPS), outer membrane (OM), periplasmic space (PPS), inner membrane (IM), peptidoglycans (Pep)
(B) Structure of LPS.

The membranes of *H. pylori* consist among others of simple lipids (such as free fatty acids, triglycerides and cholesterol) and phospholipids (see Table 2 for phospholipid composition).[70] The fatty acid composition of *H. pylori* is uncommon and distinguishes it from the closely related *Campylobacter jejuni*.[69] As *H. pylori* is most likely not able to synthesize cholesterol, it is dependent on external supply.[71] The bacteria can extract cholesterol from the host cell and integrate it into their membrane after it has been α-glycosylated by its cholesterol-α-glucosyltransferase (Cgt).[72]

H. pylori´s LPS is quite exceptional in many ways. It is highly modified to evade the host´s immune response and shows a low endotoxicity as compared to those of other pathogens. It is therefore considered an important virulence factor in the persistent colonization of the human stomach.[39] The O side chains of *H. pylori´s* LPS are diversely modified in a way to resemble Lewis blood group antigens and therefore serve as molecular mimicry.[73] Interestingly, the way of *in vitro* bacterial cultivation has an effect on LPS: When bacteria were grown in liquid culture the normal high-molecular weight LPS (S-LPS) is produced. Cultivation on solid media on the other hand results in the loss of the O side chain and consequently in low-molecular weight LPS (R-LPS).[74] Due to modifications in the phosphorylation and acylation pattern (underphosphorylation and -acylation) *H. pylori´s* lipid A is less negatively charged, and therefore more resistant to CAMPs and less biologically active.[39,40] Lipid A can also be modified by a phosphoethanolamine transferase and consists of longer fatty acid chains as compared to other bacteria.[75,76]

Table 2 Phospholipid composition of *Helicobacter pylori´s* membrane

The table shows the phospholipid composition of the membranes of *H. pylori* according to different authors.[70,77,78] The results are, however, inconsistent.
The following phospholipids are listed: Phosphatidylserine (PS), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidic acid (PA), Cardiolipin (DPG/CL), Lysophospholipids (L-).
Dyed in green = phospholipid present, dyed in red = phospholipid absent.

PS	PE	PC	PG	PA	DPG/CL	L-PE	L-PC	L-PG	*Ref.*
+	+	+	-	+	-	+	-	-	70
3-4 %	12-69 %	4-8 %	2-4 %	-	7-10 %	2-39 %	-	-	77
-	63-72 %	0.9-2 %	3-10 %	-	10-18 %	6-10 %	0.3-1.5 %	1 %	78

1.2 Annexins

1.2.1 Structure and membrane binding

Annexins are a multigene protein superfamily and are known for their ability to bind to membranes in a calcium-dependent way. Discovery of annexins started in the late 1970s/ early 1980s and up to date, there are twelve known annexin subfamilies in mammals, Annexin A1-A11 and A13.[79,80] Annexins consist of a core domain and an N terminal domain (see Figure 4).

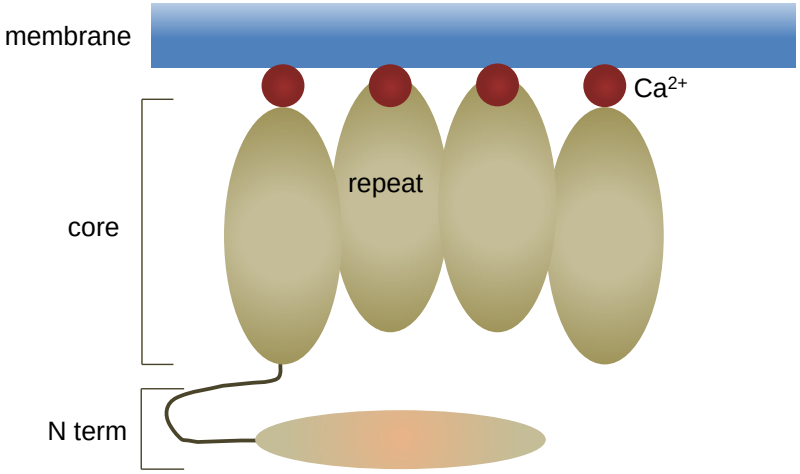


Figure 4 Structure of annexin proteins

Simplified scheme of the structure of a membrane bound annexin protein. The protein consists of a conserved protein core domain (core) with four homologous annexin repeat domains (repeat) and a variable N-terminal domain (N term). Annexin is bound to the membrane through calcium ions (Ca^{2+}).
Adapted from Gerke, V. et al. [81]

The core is highly conserved and consists of four homologous, mainly α-helical domains.[82] The core domain is the part of the protein responsible for the membrane binding.[81] Binding occurs via ten to twelve calcium ions to the head groups of polar phospholipids in the membrane and is reversible. As annexins are mainly cytosolic, they usually bind to phospholipids on the inner leaflet of the eukaryotic membrane. They show a high affinity to the negatively charged phosphatidylserine (PS) and slightly lower to phosphatidyl-ethanolamine (PE), though some annexins may also bind to phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI) or other membrane lipids.[82,83] *In vitro* studies additionally showed a connection between membrane cholesterol and annexins. For Annexin A6 a direct, calcium-independent binding to cholesterol has been described, while for other annexins (including Annexin A2 and A5) a mediating effect of cholesterol has been shown.[84,85] For the latter, the presence of cholesterol increased the calcium-dependent membrane binding to phospholipids.[85]

In contrast to the conserved core, the N-terminal domain shows a great variety in length as well as in amino acid sequence. It plays an important regulatory role both for the function of the core component and for the interaction with other proteins.[79] This interaction with other proteins mainly takes place with cytosolic ligands, for example with proteins from the S100 family,[81] but it has been proposed that membrane association could also be mediated via protein ligands (e.g. for Annexin A5 interacting with the plasma membrane of platelets).[86]

Annexin A5 (ANXA5), which was predominantly used in this study, was described as "endonexin II" in 1987 (see 6.2 for alternative names of ANXA5).[87] It has a very short N-terminus of less than twenty amino acids.[82] ANXA5 has been reported to have a high binding affinity to the membrane phospholipids PS, PE and PG, a lower affinity to specific PA and PI, and none to phosphatidylcholine (PC).[88] A calcium-independent lipid binding has also been described for ANXA5: a conformational change in acidic pH enables the protein to insert itself in PS-rich monolayers.[82] Moreover, ANXA5 is able to form two dimensional crystal-like arrays on phospholipid-containing membranes via a trimeric self-assembly.[89]
(Also see Figure 33 for the different annexin-membrane interactions.)

1.2.2 Functions of annexins

Annexins are soluble proteins and mainly occur in the cytosol, but Annexins A1, A2, A4 and A5 have been found extracellularly (both on the cell surface and in the blood circulation), though lacking a 5′-leader sequence, which normally serves as secretory signal.[90-92]

The name of the annexin group originates from the Greek word "annex", which translates as "bring/hold together"[80].[80] This terminology points to one of the major functions of annexins, the interaction with membranes. Other proposed functions are a role in membrane trafficking and organization, including exo-, endo- and phagocytic processes (see Figure 5).[80]

When membrane-bound, annexins can serve both as ion channels and as regulators of ion channels, though the exact mechanisms remain unclear.[80] Additionally, annexins interact with various signalling pathways; ANXA5 for example can inhibit protein kinase C.[93] ANXA5 can also interact with the actin-component of the cytoskeleton of activated platelets.[94] Other annexins show association with cytoskeletal parts, too, and serve modulatory functions there.

The property of ANXA5 to form two-dimensional arrays enables it to stabilize the membrane and the cell´s shape, serving as a scaffold, and to promote the repair of defect cell membranes.[82,95]

Extracellularly, annexins were found to interact with bacteria and viruses (see 1.2.4) as well as with components from the extracellular matrix, and seem to play a regulatory role in inflammation, coagulation and fibrinolysis.[82]

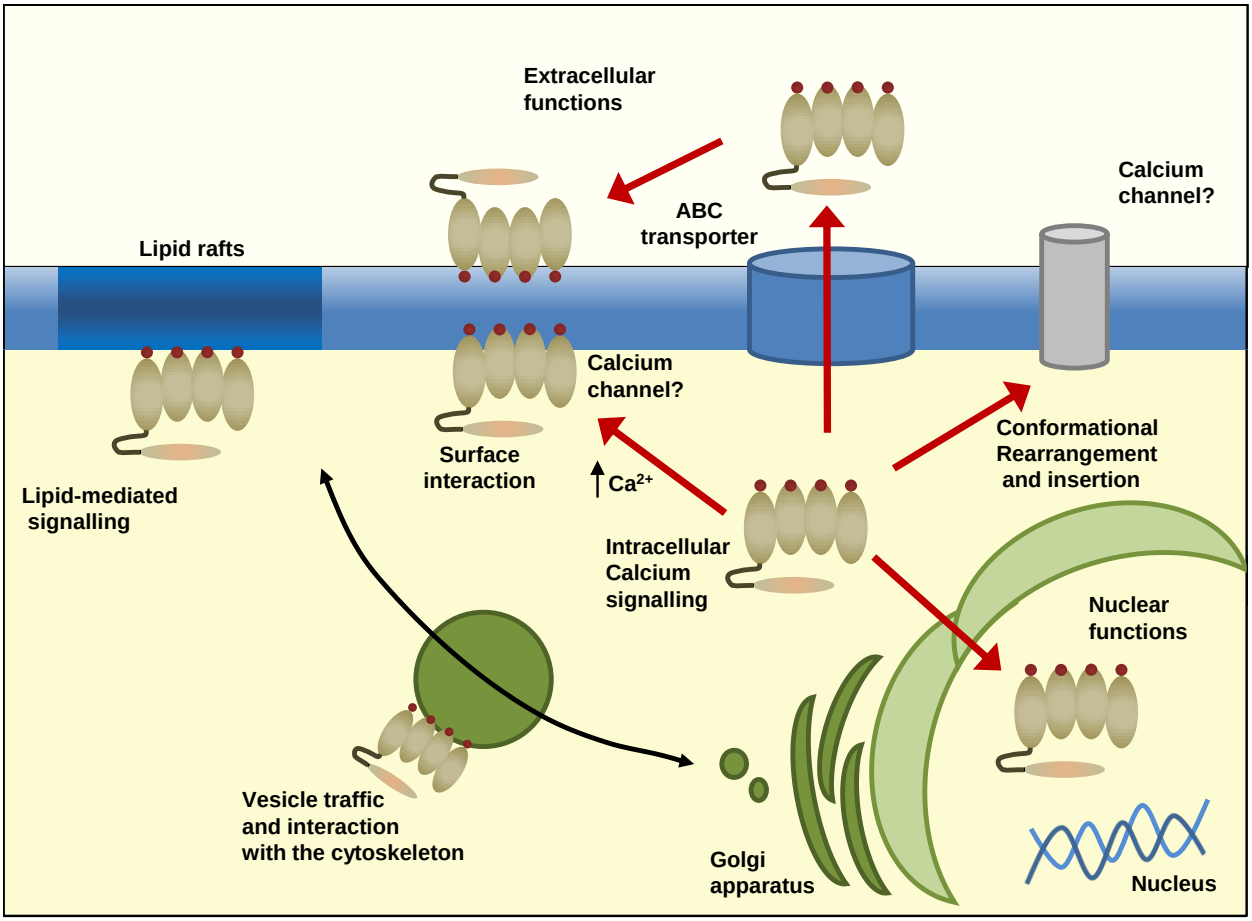


Figure 5 Overview of annexin functions

A variety of extra- and intracellular functions have been proposed for members of the annexin family. As can be seen in the figure above, functions include a role in intracellular and lipid-mediated signalling, vesicle trafficking, interaction with the cytoskeleton, nuclear functions and function as ion channel.
Adapted from Lizarbe MA et al.[82]

1.2.3 Annexins and disease pathology

There is a great variation in the distribution and expression level of different annexins in the human body. Some annexins (including Annexin A1, A2 and A5) can be found in a variety of different tissues and are present in abundant numbers, whereas others are restricted to certain tissue types.[96] Annexins A1, A2, and A5 all occur in the gastrointestinal tract and Annexins A2 and A5 are present abundantly in different cell types of the stomach, though mainly in glandular cells.[97]

Many *in vitro* functions have been described for members of the annexin family (see 1.2.2), but *in vivo* functions remain poorly understood. Nevertheless, the presence or absence and the up- or downregulation of annexins seem to play a role in a variety of disease pathologies ("annexinopathies"[98]).[98]

ANXA5 is proposed to have an antithrombotic role and is considerably reduced in patients with the antiphospholipid syndrome, a disease being characterised by thrombotic events and habitual abortions.[99] Additionally, anti-ANXA5 antibodies were found in patients with early recurrent pregnancy losses as well as with other autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis.[100-102]

Annexins can play a role in cancer development, progression and metastasis as well as in drug resistance of various cancer types.[103-105] In the following, the focus will be laid on the connection between members of the annexin family and gastric cancer:
ANXA5 is associated with the multidrug resistance (MDR) of gastric cancer and shows a positive correlation with the upregulation of MDR protein.[103] A significant association was also shown between annexin A1 (ANXA1) and peritoneal metastasis as well as with serosal invasion in gastric cancer, and high ANXA1 expression seems to be a risk factor for a worse outcome.[106] Annexin A2 (ANXA2) and A4 are up-regulated in gastric cancer and are especially overexpressed in *H. pylori*-infected tumour tissues.[107]

1.2.4 Interaction of annexins with microorganisms

1.2.4.1 Interaction of annexins with viruses and protozoa

Some annexins are able to interact with viruses and can therefore have an influence on the infection process.[82] ANXA5 was shown to bind calcium-dependently to an envelope protein of the Hepatitis B virus and to inhibit the binding of the viral protein to human hepatocytes as a consequence.[108] In contrast, it was proposed that some annexins might play a role in promoting virus infection, serving as a receptor on the cell surface or enhancing membrane fusion. The interaction between ANXA5 and the influenza virus seems to be essential for a successful infection.[109] The same was stated for ANXA2 interacting with the human cytomegalovirus (CMV), the human papilloma virus type 16 (HPV16) and enterovirus 71 (EV71).[110-112] Moreover, promastigotes of *Leishmania donovani* can bind ANXA5.[113]

1.2.4.2 Interaction of annexins with bacteria

In 2012 Rand et al. published that ANXA5 was able to bind to some gram-negative bacteria (*Pseudomonas aeruginosa*, *Shewanella putrefaciens* and *Haemophilus influenzae*), but not to the tested gram-positive bacteria (*Enterococcus faecalis*, *Streptococcus pyogenes* and *Streptococcus agalactiae*). Their data showed that ANXA5 bound calcium-dependently to the

lipid A core domain of the LPS, an important structure of the membrane of gram-negative bacteria (see Figure 3). Both *in vitro* and *in vivo* experiments displayed a possible role of ANXA5 in inhibiting the effect of LPS in endotoxin activities.[114] In contrast, ANXA1 and ANXA2 were found to bind only to the lipid A component, but not to the whole LPS.[115]

1.2.5 Technical applications of Annexin A5 in laboratory work

The membrane phospholipid PS is mainly located in the inner leaflet of cells (trans-bilayer asymmetry of PS).[116] This is achieved through active recruitment by different lipid transporters, flippases and floppases.[116,117] During early apoptosis (programmed cell death) these enzymes are deactivated, the asymmetric distribution is lost and PS is presented on the outside of the cell.[116] There it serves as a recognition and clearance signal for phagocytes to detect apoptotic cells.[117]

ANXA5 is frequently used in laboratories to detect early apoptotic cells, as it is binding to the then externalized PS. The cells can be labelled with fluorophore-coupled ANXA5 and then quantified by flow cytometry or visualized via microscopy.[118] Additionally, various studies over the last decades have suggested a potential use of labelled ANXA5 for *in vivo* imaging, ranging from the detection of arterial thrombi with gamma camera imaging to the identification of apoptotic cells in MR or nanoSPECT/CT imaging.[119-121]

1.3 Aims of this study

Considering the vast socioeconomic impact of *H. pylori* infections and its increasing drug resistance, it is crucial to carry out further research to get a better understanding of the pathophysiological processes of *H. pylori* infections and to identify and analyse unknown influencing factors on them.[122,123] In the long run this will help to develop new preventive, diagnostic and therapeutic options.

Preliminary microscopic observations in our laboratory showed a potential interaction between *H. pylori* and the human protein ANXA5. While annexins are present in the human stomach, little is known about their physiological function or their relevance for the *H. pylori* infection and pathogenicity, though some connections between annexins and gastric cancer have been previously published.[97,105-107,124]

This study was hence conducted to confirm and define an interaction of *H. pylori* and ANXA5. First, a specific binding assay had to be developed. Then, the nature of the binding had to be characterized and potential implications on *H. pylori´s* pathogenicity factors had to be investigated. Additionally, the interaction between various other bacterial species and ANXA5, as well as between *H. pylori* and different annexins, had to be tested. Finally, in order to analyse the potential role of annexins in complex infection processes and to get a better understanding of the physiological relevance of the binding, human gastric tissue samples had to be analysed.

2 Material and Methods

2.1 Material

2.1.1 Bacterial strains

2.1.1.1 Helicobacter pylori strains

All *H. pylori* strains used for this study are listed in the table below.

Strain	***Description***	***Reference***
26695	*H. pylori* wildtype	[125]
G27	Clinical isolate from Grosseto hospital in Italy	[126]
P12	Clinical isolate (888-0) from the Department of Medical Microbiology and Immunology, University of Hamburg, Germany	[127]
P12 GFP	P12wt with pHel4-GFP plasmid; expresses GFP cytoplasmic Cam^R	[128]
P12Δ*cagA* GFP	P12Δ*cagA* with pHel4-GFP plasmid Cam^R	Luisa F. Jiménez Soto, unpublished
P12Δ*cgt*	P12 strain with *cgt* gene removed by deletion of ORF by recombination and replacement with chlor-amphenicol cassette. Original plasmid made by the lab of cooperation partner Barry Marshall, Australia.	Luisa F. Jiménez Soto, unpublished
P12Δ*PAI* GFP	P12Δ*PAI* with pHel4-GFP plasmid Cam^R	Luisa F. Jiménez Soto, unpublished
P12Δ*vacA* GFP	P12Δ*vacA* with pHel4-GFP plasmid Cam^R	Luisa F. Jiménez Soto, unpublished
P12 Δ*vacA* Δ*cagA* GFP	P12Δ*vacA* GFP with pJP52a/b plasmid [129] Cam^R Kan^R	This study
P145	ATCC 45526	[130]
P217	Clinical isolate	[131]
Tx30a	Clinical Isolate; *cagA*-negative ATCC 51932	[37]
X47	Mouse adapted strain of *H. pylori (feline origin)*	[132]

2.1.1.2 Other bacterial strains

All non-*H. pylori* bacterial strains used for this study are listed in the table below.

Species/ Strain	***Description***	***Reference***
Bacillus subtilis BD 170	EW-X 328, ATCC 33608	American Type Culture Collection (ATCC®)
Bacillus subtilis BD 630	EW-X 328	[133]
Campylobacter jejuni	ATCC 81176	American Type Culture Collection (ATCC®)
Escherichia coli DH5α	*E. coli* F-Φ80d *lacZ* Δ*M15* Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17	Invitrogen
Escherichia coli EPEC	Enteropathogenic *E. coli*, clinical isolate #5563 from Max von Pettenkofer-Institute, LMU Munich	[128]
Escherichia coli UPEC	Uropathogenic *E. coli*, clinical isolate #6011 from Max von Pettenkofer-Institute, LMU Munich	[128]
Lactobacillus acidophilus	EW-118, NCC-12	Nestlé, Lausanne
Lactobacillus johnsonii	EW-114, NCC-1680	Nestlé, Lausanne
Moraxella catarrhalis	ATCC 25238 not binding to CEACAMs	[134]
Moraxella catarrhalis	ATCC 43617 binding to CEACAMs 1,3,5	[134,135]
Neisseria gonorrhoeae	N356 (= MS11 strain), ERM^R	TF Meyer, Berlin
Neisseria gonorrhoeae	N302 not binding to CEACAMs	[136]
Neisseria gonorrhoeae	N309, MS11 strain, constitutively producing OPA_{52} binding to CEACAMs 1,3,5	[135,136]
Staphylococcus aureus	ATCC 29213	American Type Culture Collection (ATCC®)
Streptococcus pneumoniae	ATCC 49619	American Type Culture Collection (ATCC®)

2.1.2 Cell lines

All cell lines used for this study are shown in the table below.

Cell line	***Description***	***Reference***
AGS	Human gastric adenocarcinoma cell line ATCC CRL-1739	[137]

2.1.3 Human tissue

All human tissue was obtained commercially in the form of paraffin embedded tissue on object slides.

Tissue	***Reference***
Stomach, Antrum, normal	American master tech, CSS0625P
Naturally occurring *H. pylori* in gastric tissue	American master tech, CSH0125P
Placenta, normal	American master tech, CSP035P

2.1.4 Reagents and solutions

2.1.4.1 Reagents

Brucella broth (BD Falcon), Acrylamide/Methylenbisacrylamide 30 % (29:1) and X-Gal (Roth), Streptomycin, Trimethoprim, Vancomycin, Nystatin, Ampicillin, Phrobol-12-myristat-13-acetate PMA, Cytochalasin D, Ethidium Bromide, Paraformaldehyde, Glutaraldehyde, Pepstatin, Triton X-100, Tween 20, DMSO (Heptakis), Chloramphenicol (Serva), Kanamycin, Phenyl-methylsulfonylfluorid PMSF (Merck), GC Agar, LB Agar, LB broth (Oxoid), RPMI 1640 (life technologies/Thermo Fisher Scientific), Phenol red (phenolsulfonphthalein) (Sigma), Trypan blue (Sigma), HEPES buffer solution 1M (gibco), DAPI (Sigma), Annexin A1 (R&D Systems), Annexin A2 (abcam), Annexin A5 Alexa Flour® 488/555/594/647 (life technologies/Thermo Fisher Scientific), recombinant human Phalloidin TexasRed/ Alexa Fluor® 647 (Molecular Probes), Millipore Immobilon Western (Millipore), Methanol (Roth), Ethanol (Roth), PI (MACS Miltenyl Biotech), Xylene (Roth)

2.1.4.2 Solutions and buffers

2x Single gel system buffer	152 mM Tris/HCl; 0.2 M serine; 0.2 M glycerol; 0.2 M asparagine; pH 7.4
2x SDS loading buffer	100 mM Tris HCl pH 6.8; 4 % SDS; 0.2 % Bromophenol blue; 20 % Glycerol; 10 % β-Mercaptoethanol (optional)
5x SDS loading buffer	10 % SDS; 0.5 M Tris HCl (pH 6.8); 50 % Glycerol; 5 % Bromophenol blue
50x TAE buffer	242 g/l Tris Base; 57.1 ml/l Glacial Acetic Acid; 50 mM EDTA
Agarose loading buffer 6x	0.25 % Bromophenolblue; 0.25 % Xylene Cyanol FF; 30 % Glycerol; in TAE buffer
Annexin A5 binding buffer (AnxBuf)	10mM Hepes; 150mM NaCl; 5mM KCl; 5mM $MgCl_2$; 1.8mM $CaCl_2$; pH 7.4; sterile filtrate and store at 4°C *(modified after Kenis at al.[138])*
Annexin A5 binding buffer without Hepes	150mM NaCl; 5mM KCl; 5mM $MgCl_2$; 1.8mM $CaCl_2$; pH 7.4; sterile filtrate and store at 4°C
Antibody purification – Binding buffer	20mM Sodium Phosphate pH 7.0
Antibody purification – Elution buffer	0.1 M glycine-HCl pH 2.7; important to prepare fresh
Antibody purification – Neutralization buffer	1M Tris-HCl pH 9.0
Complete medium (CM)	RPMI 1640 complemented with 10 % heat inactivated foetal calf serum (FCS)
ELISA buffer	50mM Tris/ HCl pH 7.6
ELISA – Blocking buffer	10 % FCS in PBS
ELISA – Coating buffer	100mM Na_2HPO_4; pH 9.6
ELISA – Reaction-stopper	1M H_2SO_4
ELISA – Washing buffer	PBS-Tween-20 0.05 %
EM-fixing buffer	0.2M HEPES; 1 % Glutaraldehyde; store at 4°C
EM-storage buffer	0.2M HEPES; store at 4°C
PBS 10x	2 g/l KCL; 80 g/l NaCl; 2 g/l KH_2PO_4; 14.4 g/l Na_2HPO_4 (29 g/l $Na_2HPO_4 \cdot 12H_2O$)
PBS*	PMSF 1:100 in PBS

SDS running buffer EP	250mM Glycin; 25mM TrisHCl; 0.1 % SDS; pH 8.3
Vitamin Mix	100 g/l a D-Glucose; 10 g/l L-Glutamine; 26 g/l L-cysteine; 0.1 g/l Cocarboxylase; 20 mg/l Fe(III)-Nitrate; 3 mg/l Thiamine; 13 mg/l p-Aminobenzin acid; 250 mg/l Nicotinamide-adenine dinucleotide (NAD); 10 mg/l Vitamin B12; 1.1 g/l L-cysteine; 1 g/l Adenine; 30 mg/l Guanine; 0.15 g/l L-Arginine; 0.5 % Uracil
Western blot - Anode buffer I	0.3M Tris-HCl; 10 % Methanol; pH 10.4
Western blot - Anode buffer II	25 mM Tris-HCl; 10 % Methanol; pH 10.4
Western blot - Blocking solution	3 % BSA in TBS-Tween-20 0.075 %
Western blot - Cathode buffer	25 mM Tris-HCl; 40mM 6-amino-n-caproic acid (or glycine); 10 % Methanol; pH 9.4
Western blot - Stripping solution	25 mM glycine-HCl; 1 % SDS; pH 2
Western blot - Washing buffer	Tween-20 0.075 % in TBS TBS = 150 mM NaCl; 20mM Tris-Cl; pH 7.5

2.1.5 Antibodies

2.1.5.1 Primary antibodies

α-ANXA1	Goat polyclonal antibody against human/mouse/rat Annexin A1 (R&D Systems)
α-ANXA2	Goat polyclonal antibody against human/mouse/rat Annexin A2 (R&D Systems)
α-ANXA2	Rabbit monoclonal antibody against human/mouse/rat Annexin A2 (abcam)
α-ANXA5	Mouse monoclonal antibody against full length human Annexin A5 (Santa Cruz Biotechnology)
α-CagA (AK 299)	Rabbit antibody against a peptide with EPIYA motifs (Fischer &Haas)[139]
α-*H. pylori* (AK 175)	Rabbit polyclonal antibody against a soluble extract of *H. pylori* P1 strain
α-human IL-8 (coating ab ELISA)	Mouse monoclonal antibody against human IL-8 (BD Pharmingen)
α-human IL-8 (detection ab ELISA)	Mouse monoclonal antibody against human IL-8, biotinylated (BD Pharmingen)
α-Phosphotyrosin (4G10)	Mouse monoclonal antibody against tyrosine phosphorylation, clone 4G10 (Upstate Millipore)

α-RecA (AK 263)	Rabbit polyclonal antibody against RecA of *H. pylori* P1 strain[140]
α-Tubulin	Mouse monoclonal antibody against human and mouse tubulin alpha subunit (Upstate)
α-VacA (AK297)	Rabbit antibody against native whole *H. pylori* 60190 VacA[141]

2.1.5.2 Secondary antibodies

α – goat IgG $Alexa_{647}$	Alexa Fluor®$_{647}$-conjugated donkey antibody against goat IgG (H+L) (life technologies)
α – goat IgG POX	Rabbit peroxidase-conjugated polyclonal antibody against goat IgG (Sigma)
α – mouse IgG POX	Goat peroxidase-conjugated monoclonal antibody against mouse IgG (Sigma)
α – rabbit IgG $Alexa_{488}$	Alexa Fluor®$_{488}$-conjugated donkey antibody against rabbit IgG (H+L) (life technologies)
α – rabbit IgG $Alexa_{647}$	Alexa Fluor®$_{647}$-conjugated goat antibody against rabbit IgG (H+L) (Invitrogen)
α – rabbit IgG POX	Goat peroxidase-conjugated monoclonal antibody against rabbit IgG (Sigma)

2.1.6 Plasmids

pJP52a/b*	pBluescript with insertions of flanking regions of CagA with a Kanamycin cassette using BamHI and Xhol

** a is for antisense insertion of the cassette and b for sense insertion*

2.1.7 Oligonucleotides

JP17	ACCGCTCGAG TTACCACTAG CCCTAAAG
JP18	CGGGATCCTA GCCACTTCTC TTTTTG

2.1.8 Commercially available kits

QIAamp DNA Mini Kit (Quiagen)	for isolation of genomic, mitochondrial, bacterial, parasite or viral DNA

2.1.9 Equipment

PAGE-Mini Gel System and Voltage Units Power Pac 300 and Power Pac 1000 (Bio-Rad), Incubator (Binder), Incubator *Ultima* (Revco), Microincubator MI22N (Scholzen), Incubator HERAcell150i (Thermo scientific), ChemiDoc MP System (Bio-Rad Laboratories), Trans-illuminator (Bio-Rad), BD FACS Canto II Flow Cytometer (BD Bioscience), Spectrophotometer DR/2000 (Hach), CLARIOstar Microplate reader with microinjection system (BMG Labtech), Multichannel Pipette (MATRIX Corporation), Agarose Gel Electrophoresis chamber (Bio-Rad), Centrifuge Megafuge 16R and Megafuge 3.0 R (Heraeus), Centrifuge Mikro 20 (Hettich), Centrifuge 5424R (eppendorf), Magnetic Stirrer MR 3000 (Heidolph), Microscope DM IRB (Leica), Confocal Microscope SP5/ SP8 with STED (Leica) and TCS Software (Leica), Confocal microscope (BX61, Olympus), Spinning disc microscope and Volocity software (Perkin Elmer), PCR machine T3 Thermocycler (Biometra), Scales (Fischer Biotech), pH Meter (WTW), Semi-dry Blotting Chamber (Fischer Biotech), Sterile Hood (BDK), Vacuum Centrifuge Speed-Vac DNA 110 (Savant), Vortex Genie 2 (Scientific Industries), Water Bath (GFL)

2.1.10 Consumables

ELISA Maxisorp plates (Nunc), 6-, 12-, 24-, 48-, 96- well plate (Falcon), Cell scrappers (Falcon), FACS tubes (Becton Dickinson), Eppendorf safelock tubes (Eppendorf), Falcon tubes (Sarstedt), Freezing Tubes 2 ml (Nalgene), PVDF membrane (Bio-Rad), Precision Plus Protein Allblue standards marker (Bio-Rad), 0.2µm- Sterile filters (Millipore), Cell culture treated flasks (75 cm^2) (BD Falcon), Filter paper (Whatman), glass bottom culture dish uncoated/coated with Poly-d-Lysine (MatTek Corporation), Foetal Horse Serum (PAA Laboratories GmbH), Bovine Serum Albumin BSA (Sigma), RPMI-1640 (Invitrogen/GIBCO BRL), Foetal Calf Serum FCS (Invitrogen/GIBCO BRL), Trypsin-EDTA (TE), Dulbecco PBS ($+Ca^{2+}$, $+Mg^{2+}$) and Dulbecco PBS ($-Ca^{2+}$, $-Mg^{2+}$) (Invitrogen/GIBCO BRL), DMSO, EGTA and EDTA (SIGMA Aldrich), Proteinase K (Sigma), Agarose (Roth), Nucleotide ladder, 10 kb (Fermentas/ Thermo), HiTrap™ Protein G HP, 1 ml (GE Healthcare Life Sciences), pooled normal human serum (Innovative Research), ProLong Gold Antifade Mountant (Thermo scientific)

2.2 Methods

2.2.1 Bacterial culture and work

2.2.1.1 Culture of all bacterial strains

Bacterial strains were defrosted from -70°C by scrapping some of the frosted material with an inoculation loop and passing it on to a GC agar plate (without antibiotics). *H. pylori* and *Campylobacter jejuni* cultures were left in the incubator for 3 days to grow and afterwards passed to a new GC agar plate (complemented with their respective antibiotic) every day. After 2 passages or more, bacteria were used for experiments.

All other bacterial strains used in this work where defrosted and from the next day on passaged every day for 2 days at the minimum before used for experimental purposes.

All bacterial strains grew at 37°C on different agar plates and with different atmospheres, as shown in the following table.

Bacterial strain	***Agar plate***	***Atmosphere***
Bacillus subtilis	Columbia blood agar	normal atmosphere
Campylobacter jejuni	Serum	10 % CO_2-atmosphere
E. coli	Columbia blood agar	normal atmosphere
H. pylori (exception see below)	Cholesterol[142]	85 % N_2, 10 % CO_2 and 5 % O_2
H. pylori P12Δcgt	Serum	85 % N_2, 10 % CO_2 and 5 % O_2
Lactobacillus acidophilus	Columbia blood agar	10 % CO_2-atmosphere
Lactobacillus johnsonii	Columbia blood agar	10 % CO_2-atmosphere
Moraxella catarrhalis	Columbia blood agar	normal atmosphere
Neisseria gonorrhoeae N302/N309	Columbia blood agar	10 % CO_2-atmosphere
Neisseria gonorrhoeae N356	Serum	10 % CO_2-atmosphere
Staphylococcus aureus	Serum	normal atmosphere
Streptococcus pneumoniae	Columbia blood agar	5 % CO_2-atmosphere

For some experiments *H. pylori* was also grown on serum or Columbia blood agar plates; this will be specially indicated in the text.

Agar Type	***Description***
GC Agar serum	36 g/l GC agar, 8 % horse serum, 1 % Vitamin mix
GC Agar cholesterol	36 g/l GC agar, 1x cholesterol (GIBCO), 1 % Vitamin mix
Blood agar	Columbia agar with sheep blood (Oxoid)

Antibiotics for the growth of *H. pylori* strains were used in the following final concentrations:

Chloramphenicol 6 mg/l

Kanamycin 8 mg/l

2.2.1.2 Liquid culture of Helicobacter pylori

For some experiments *H. pylori* that had been grown in liquid culture was used. After defrosting and incubation for 3 days on GC agar plates *H. pylori* was passaged and on the following day resuspended in PBS. OD_{550} was measured (see 2.2.1.4). A well from a 6-well plate was prepared with 2 ml Brucella broth and 8 µl Cholesterol 250x, and bacteria were added in an OD_{550} of 0.075/ml. After approximately 24 hours incubation at 37°C in a 10 % CO_2-atmosphere, the bacteria were used for experiments. Vitality was checked under the microscope, and OD_{550} was measured before use.

2.2.1.3 Freezing of bacteria

After a minimum passage time of 3 days, bacteria were collected from one or half a plate with a cotton swab and resuspended in 1 ml of freezing media. *H. pylori*-freezing medium was used for all strains except for *Neisseria gonorrhoeae* N302 and N309, for which a special *Neisseria gonorrhoeae*-freezing medium was used. Storage was performed at -70°C.

Helicobacter pylori-freezing medium	10 % FCS (not heat inactivated), 20 % Glycerol, 70 % BB (Brucella broth), sterile filtrated
Neisseria gonorrhoeae-freezing medium	20 % Glycerol, 80 % LB (Luria-Bertani Broth), sterile filtrated

2.2.1.4 OD_{550} measurement

Before bacteria cultures were used for experiments, optic density (OD) was measured in a photometer at a wavelength of 550 nm to determine the approximate number of bacteria.

Bacteria were collected from the agar plate with a cotton swab, resuspended in PBS and measured against a blank value. An OD_{550} of 0.1 is equivalent to approximately $3x10^7$ CFU/ml of *H. pylori*.

2.2.1.5 Transformation of Helicobacter pylori

With the method of transformation exogenous DNA can be inserted in bacteria. *H. pylori* is naturally competent,[143] meaning that it takes up DNA without being artificially stimulated.

A cholesterol agar plate was prepared by adding 1-2 µl of the plasmid solution (maximum concentration of 200 ng/µl) to one spot. A small amount of *H. pylori* was added to the same spot. After approximately 6 hours of incubation everything that had grown was passaged to a selective agar plate containing the corresponding antibiotics to the plasmids resistance. After 1 day of incubation a single clone was picked with a sterile pipette tip and again transferred to a selective plate. After further passaging the clone was frozen at -70°C.

2.2.1.6 Preparation of samples for electron microscopy

H. pylori was incubated for 1 hour at 37°C in 5 % CO_2 in a cell culture treated flasks filled with complete medium with an OD_{550} of approximately 1.8. After harvesting, centrifugation was done at 4,000 rpm at 4°C for 5 minutes. The supernatant was discarded and after washing steps the pellet was resuspended in 1 ml of EM-fixing buffer. After 1 hour incubation at RT the sample was centrifuged again at 4,000 rpm at 4°C for 10 minutes, the supernatant discarded and the sample was stored in EM-storage buffer at 4°C (see Figure 1 for exemplary results).

2.2.1.7 Annexin binding assay

2.2.1.7.1 Annexin binding assay

Bacteria were resuspended and added in an OD_{550} of 0.1 (unless stated otherwise) in an Eppendorf tube with 1 ml RPMI 1640 and 1.5 µl or 2.5 µl of ANXA5. The ANXA5 was fluorophore-coupled if needed, with Alexa Fluor® 488, 555, 594 and mostly with 647. After 1 hour incubation at 37°C in 10 % CO_2 the samples were put on ice (if the bacteria were not used for further infections). The samples were then treated depending on the further processing for various analyses. The assay could also be performed with corresponding amounts of ANXA1 or ANXA2.

2.2.1.7.2 Flow cytometry analysis

The samples (see 2.2.1.7.1) were washed twice in Annexin A5 binding buffer with centrifugation at 4,000 rpm at 4°C for 5-10 minutes preparing them for flow cytometry

analysis. The pellets were then kept at 4°C. Right before measurement in the flow cytometer the pellet was resuspended in Annexin A5 binding buffer (optionally with PI 1:100 or DAPI 1:10,000). The binding of ANXA5 to the bacteria was measured as the increase of the fluorescence in the channel of the fluorophore coupled to ANXA5 (mostly Alexa Fluor® 647).

For the analysis of the data see 2.2.6.1.

2.2.1.7.3 Microscopy

For imaging, PFA (to a final concentration of ~2.5 %) was added to the samples (see 2.2.1.7.1) right after incubation. They were left for a minimum of 15 minutes at RT and then stored at 4°C. Optionally DAPI staining was performed (DAPI was added 1:10,000 for 10 minutes at RT with a washing step before and after staining). To transfer the samples on an object slide, cover slides were laid in wells of a 24-well plate and the samples were added. After centrifugation at 3,000 rpm at 4°C for 20 minutes the samples on the cover slides could be mounted with mounting medium on an object slide. After drying for at least 24 hours imaging could be performed. The object slides were stored in the dark at RT.

2.2.1.7.4 Western blot analysis

For analysis with Western blot the samples (see 2.2.1.7.1) were washed twice in Annexin A5 binding buffer with centrifugation at 4,000 rpm at 4°C for 5-10 minutes and were then resuspended in 5 µl of PBS* and 7 µl of 2x SDS loading buffer. The samples were boiled for 10 minutes in a waterbath with 95°C and stored at -20°C until usage (for general Western blot protocol, see 2.2.4).

2.2.2 Cell culture and infections

2.2.2.1 Culture of AGS cells

AGS cells were cultivated in cell culture treated flasks in RPMI 1640 complemented with 10 % heat inactivated FCS (=complete medium (CM)). They were passaged every 2-3 days by splitting 1:6 to 1:8 and kept in an incubator at 37°C and 5 % CO_2. AGS cells are adherent cells and after removal of the old medium they were washed once with PBS. TE was then added to detach the cells from the bottom of the flask. After a few minutes of incubation, the cells were dissolved and could be transferred to a flask with new medium.

When cells show ~100 % confluency, there are approximately $1x10^6$ cells in one well of a 6-well plate.

2.2.2.2 Fixation of cells

Cells were fixed using PFA. The frozen 25 % PFA solution was defrosted in a heating block at 60°C. It was then added to the media of the cells creating a final PFA concentration of 2.5 %. The samples were incubated for a minimum of 15 minutes at RT and then stored at 4°C, normally in a 1 % PFA in PBS solution. Before using the fixed cells for immunostaining, washing steps were important to avoid fixation of the antibody.

2.2.2.3 AGS infections

2.2.2.3.1 Infection with *Helicobacter pylori*

AGS cells were seeded in well plates 2 days before the experiment day. Synchronisation was done (see 2.2.2.3.2). 90 % was the optimal cell confluency for conducting experiments. Bacteria were resuspended in PBS, OD_{550} was measured and the bacteria suspension was added to the cells depending on the MOI needed. The infection was incubated at 37°C in 5 % CO_2 for a certain time span (usually 1-3 hours).

Multiplicity of infection (MOI) is used to express the number of bacteria that is added in relation to one eukaryotic cell. The MOI is calculated by dividing the number of bacteria by the number of eukaryotic cells. A MOI of 60 is equivalent to the number of bacteria of OD_{550}=0.2 added to one 6-well of AGS cells (~$6x10^7$ bacteria for $1x10^6$ cells).

2.2.2.3.2 Cell synchronisation

Before an infection with *H. pylori* the AGS cells were synchronised in order to get more consistent results. The synchronisation was performed by serum deprivation. Cells enter the G_0 quiescence when they grow in medium without serum. When the medium is complemented with serum again, the cells ideally all enter the early G_1 phase simultaneously.[144]

Cells were normally cultured in CM, but on the evening before the experiment day the medium was changed to serum free RPMI. 1 hour prior to the infection the medium was changed back to CM.

2.2.2.3.3 Binding assay

AGS cells were infected (see 2.2.2.3.1) with GFP-expressing *H. pylori* in an MOI of 60. Optionally bacteria had been pre-incubated with ANXA5 (see 2.2.1.7.1) or ANXA5 was added simultaneously with the bacteria to the cells. After 1 hour of incubation the cells were put on ice, washed twice with Annexin A5 binding buffer (AnxBuf) - to remove unbound bacteria - and harvested directly before measurement in flow cytometer with a cell scraper.

The GFP-signal was measured and used to determine the number of bacteria that had bound to the cells.

2.2.2.3.4 CagA phosphorylation assay

H. pylori can inject CagA in the AGS cells where it is phosphorylated and can be detected in cell lysates.

The AGS cells were infected with *H. pylori* (see 2.2.2.3.1) with an MOI of 60. Optionally, *H. pylori* had been pre-incubated for 1 hour with ANXA5 or ANXA5 was added directly at the moment of the infection. After 3 hours of incubation the cells were put on ice and washed once with PBS*. They were harvested with a cell scraper and centrifuged at 500 g at 4°C for 10 minutes. The pellet was resuspended in 20 µl PBS* and 30 µl of 2x SDS loading buffer and boiled for 10 minutes in a waterbath. The samples were stored at -20°C (for general Western blot protocol, see 2.2.4). α-Phosphotyrosin (4G10) antibody was used 1:10,000 for detection and semi-quantitative blot analysis was performed using the Stain-Free technology.[145]

2.2.2.3.5 IL-8 ELISA

H. pylori can cause the induction of IL-8 in its host cells. The amount of IL-8 produced can be assessed with an Enzyme Linked Immunosorbent Assay (ELISA) of the supernatants of AGS cells after infection.

The supernatants from the infection for the CagA phosphorylation assay (see 2.2.2.3.4) were taken and stored in deepwell plates at -20°C. The ELISA protocol took 3 days. On the first day, the coating day, the coating antibody was mixed with ELISA-Coating buffer and pipetted on the 96-well plate, where it was incubated at 4°C for at least 8 hours. On the second day, the sample loading day, the antibody-solution was removed and 2 washing steps were performed. The plate was blocked for 2 hours at RT and then washed again twice. The samples (diluted 1:10 or 1:20 in CM) and the standard solutions were added and the plate was incubated overnight at 4°C. On the third day, the developing day, the plate was washed six times before the biotinylated α-human IL-8 antibody was added 1:1,000 in ELISA-Washing buffer + 10 % FCS. Incubation could be performed either for 45 minutes at 37°C or for 2 hours at RT and after the incubation 6 washing steps were performed. The two solutions for the Streptavidin-POX-complex were mixed in 200 µl ELISA-buffer and incubated for 45 minutes at RT before they were added to ELISA-buffer 1:50 and put on the plate. After another 45 minutes of incubation at RT and 6 washing steps the developing solution was added to the plate. Thereupon the wells changed their colour from yellow to light blue. After the three highest standard wells had turned blue, the reaction was stopped by adding

1M H_2SO_4. The results were analysed in the Clariostar plate reader. By using the standard wells the IL-8 concentration of the samples could then be calculated.

2.2.2.3.6 Annexin A2 expression in AGS cells

AGS cells were infected with the *H. pylori* strain P12 GFP or a mutant lacking the *cag*-PAI (P12Δ*PAI*GFP) in an MOI of 60 (see 2.2.2.3.1). The infection was incubated for 3 hours, the cells were put on ice and 2 washing steps were performed to remove unbound bacteria. The AGS cells were then harvested, lysates were prepared and the ANXA2 expression was analysed with Western blot immunodetection (see 2.2.4).

2.2.3 Immunohistochemistry of tissue slides

Before the tissue could be stained, deparaffinization was performed. The slides were incubated for 30 minutes at 55°C. They were then put in xylene twice for 15 minutes and rehydrated through a graded alcohol series (10 min 100 %, 5 min 96 %, 2 min 80 %, 1 min 70 %, 1 min 50 %, dip in H_2O_d). Antigen retrieval was obtained by boiling the samples in citrate buffer three times for 5 minutes. After washing in PBS the slides were blocked in 1 % BSA and 2.5 % goat serum for 1 hour at 37°C. 0.5 % saponin was added to the blocking solution to permeabilize the cell membrane so that the antibody could reach their intracellular target. The primary antibody was added 1:50 in the same solution as the blocking solution and incubated for 1 hour at 37°C. After washing in PBS-tween the secondary antibody was added 1:500 in PBS-tween and 2.5 % goat serum. After 1 hour incubation at 37°C the slides were washed again and stained with DAPI (1:5,000; 2 minutes at RT). Mounting medium and glass platelets were applied and the slides were kept in the dark to dry.

The analysis was performed using the Olympus confocal microscope with the 10x objective. (For quantitative analysis see 2.2.6.3)

2.2.4 Protein work

2.2.4.1 Protein separation by polyacrylamide gel and blotting

A 6 % single gel system[146] containing trichloroethanol 0.01 % was used to separate the proteins based on their molecular weight.

The gel was prepared as follows (10 ml for two 1mm thick, 6 % gels):

2.6 ml	H_2O_{dest}
2.0 ml	Bis-Acrylamide 30 %
5.2 ml	2x single gel buffer
0.1 ml	Ammonium Persulfate
0.1 ml	Trichloroethanol
0.012 ml	TEMED

The gel polymerised and could then be stored wet at 4°C for up to 1 week.

After loading the 1 mm thick gel with 3-8 µl of the samples and the Precision Plus Protein Allblue standards marker the gel was run at 90 V for 10 minutes and at 140 V for 55 minutes in SDS running buffer. The total number of proteins was detected in the ChemiDoc MP System using the Stain-Free technology. Due to the trichloroethanol in the gel, the fluorescence of tryptophan residues of the proteins is enhanced after activation with UV-light (302 nm). The proteins can thus be visualised.[147]

In a semi-dry blotting chamber, the proteins were transferred from the gel to a PVDF membrane in 75-120 minutes at a current of 64 mA/membrane. After blotting the membrane dried for a minimum of 1 hour at 37°C or overnight at RT.

2.2.4.2 Immunodetection of proteins

The dry membrane with the proteins was activated by dipping it into methanol. It was then put into a 50 ml Falcon tube and blocked with 5 ml of Western blot-Blocking solution for 1 hour at RT. The primary antibody was added and incubated for 1 hour at RT. The membrane was washed four times (15 minutes each, in Western blot-Washing solution) before the secondary antibody in either 5 ml Western blot-Blocking solution or Western blot-Washing solution was added. After 45 minutes of incubation at RT 4 more wash steps (15 minutes each) were performed. For the chemiluminescent antibody detection Millipore Immobilon Western solution was added and the membrane was put in between two transparencies. The signal was then detected in the ChemiDoc MP System. If required further analysis was performed using the ImageLab™ software (Bio-Rad Inc.).

Alternatively, detection of fluorophore-coupled proteins in the gel could be performed by exciting and detecting the fluorophore itself in the Gel Doc documentation system.

2.2.4.3 Antibody purification

When an antibody shows a high background signal, it might be necessary to perform antibody purification. This was done using Amersham columns. These columns are coated with Protein G, which binds with a strong affinity to the F_C-terminus of IgG-antibodies.

The purification was performed according to the manufacturer´s instructions. The analysis was performed with Nanodrop (at 280 nm) and with Stain-Free analysis after Western blotting.

2.2.5 DNA work

2.2.5.1 Chromosomal DNA extraction

Bacteria were resuspended from the plate in PBS, centrifuged at 4,000 rpm at 4°C for 5 minutes and the pellet was stored at -20°C until usage.

DNA extraction and purification was then performed according to the manufacturer´s instructions (from step 3 onwards) with the QIAamp DNA mini Kit. DNA was stored in buffer from the manufacturer at -20 °C.

2.2.5.2 Polymerase chain reaction (PCR)

PCR is a molecular biological method to amplify a specific piece of DNA.[148] First, a master mix of ultrapure water and the primers was made and 9 µl were aliquoted in the PCR tubes. 1 µl of DNA template and 10 µl of a Dream-Taq-master mix (2x) (received from Desirée Plazcek) were added. After vortexing and brief centrifugation the tubes were placed in the PCR machine and the following program was run:

1. 5 min denaturation at 94°C
2. 30 sec denaturation at 94°C
3. 30 sec annealing at 50°C
4. 9 min polymerisation at 72°C

repeat steps 2.-4. 30x

5. 10 min final polymerisation at 72°C

The product was stored at 10°C overnight before further usage.

2.2.5.3 Analysis with agarose gel

For one gel 0.64 g of agarose was boiled in the microwave with 80 ml of TAE-buffer and poured into a chamber. The prepared gel could be stored in TAE-buffer overnight at 4°C.

The samples were mixed with Agarose loading buffer 6x (final volume 24 µl) and 6 µl were loaded to the gel together with a 10 kb nucleotide ladder as DNA marker. The gel was run for approximately 1 hour in TAE-buffer at 70 V and then placed in ethidium bromide for 45 minutes to dye the DNA. Results were visualised in a transilluminator.

2.2.6 Data analysis

2.2.6.1 Analysis of flow cytometry data

The data was exported from the FACS Diva software as FCS 3 file and imported in the FlowJo software (version 7.6.1). The data was analysed using the histogram of the fluorescence of interest (the emission maxima of the fluorophores used, mostly 647). A gate was created using the negative control, so that approximately 2.5 % of events of the negative control were in the positive gate (see Figure 6, left). The gate was then transferred to the samples that should be analysed (see Figure 6, right). The corresponding negative value (~2.5 %) was then subtracted from the obtained percentage and this value was used for statistical analysis as “percentage of ANXA5-positive bacteria”.

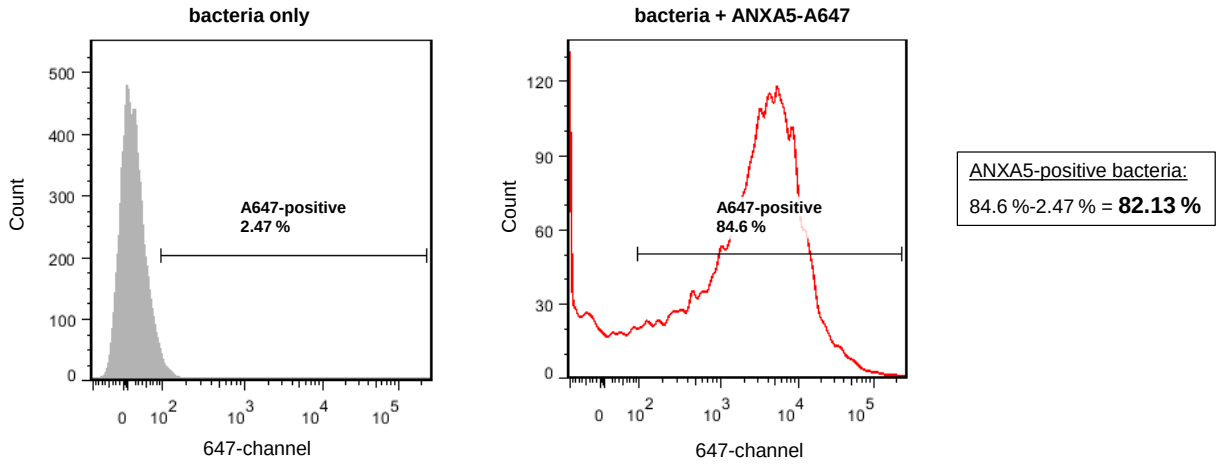


Figure 6 Gating of flow cytometry data

The control (bacteria only; grey) was gated, so that only ~2.5 % of events were in the A647-positive group. The gate was applied to the sample of interest (bacteria + ANXA5-A647; red). The percentage number of the control was subtracted from the one from the sample (as it was considered as unspecific background signal). This number was then used for graphs and statistical analysis as “percentage of ANXA5-positive bacteria”.

Sometimes it was necessary to use an additional parameter for the evaluation of the data. This was especially of interest if two samples, which showed a similar percentage of "ANXA5-positive bacteria", were analysed more closely and were compared with one another. An example with representative data is shown in Figure 7. Though the number of bacteria that bound ANXA5 is nearly the same in both populations (the percentage of "ANXA5-positive group" shows no significant difference), the curve in blue is obviously shifted to the right in comparison to the curve in red. This means that the average bacterium in the blue population bound more ANXA5 as compared to the average bacterium in the red population. To express this difference in a parameter the median fluorescence intensity (MFI) of the positively gated group was used (this value now shows the difference between the curves, see Figure 7, right).

Normally the MFI of the ANXA5-positive group was identified with the FlowJo software, but sometimes the median from all events was also directly extracted from the FACS Diva software.

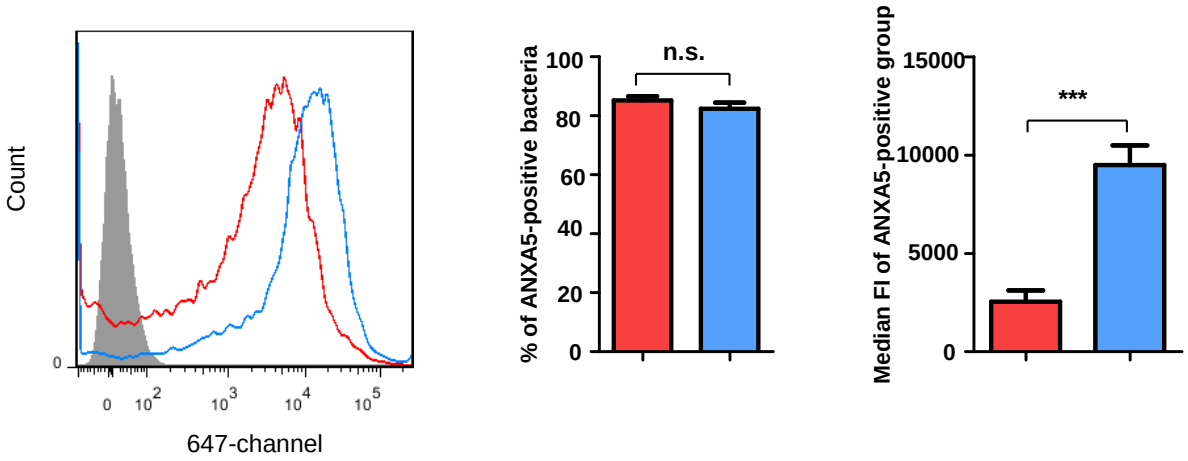


Figure 7 Use of median fluorescence intensity (MFI) as another parameter for analysis of flow cytometry data

Representative histogram shown on the left with a control (grey) and two exemplary samples (red/blue). Graphs show the percentage of ANXA5-positive events (middle) and the median fluorescence intensity of the events from the ANXA5-positive group (right).

2.2.6.2 Semi-quantitative blot analysis using the Stain-Free technology

The analysis was performed as previously described using the Image lab software (version 5.2.1).[145] The mean value of the replicates of the control was calculated. All values were divided by that mean to set the control to 100 % while still being able to do statistical analysis.

2.2.6.3 Quantitative analysis of immunohistochemistry data

Quantification of annexin expression in the tissue slides was done with the ImageJ software (Fiji) after a modified protocol from Dr. Benjamin Busch. At first the picture size was defined (x=887.5 µm, y=665.6 µm). The image (TIFF file) was cropped and the total area was measured, so that the percentage of the stained area could then be detected. To achieve this, the threshold was set in a way that the background signal in the unstained control slides (stained only with secondary antibodies) was between 0.5 and 1 %. The obtained threshold value was then used on all stained slides, which were of the same type of tissue as the control slide. All colour signals that were above the threshold were counted as annexin-positive. The sum of the annexin-positive areas was then divided by the total area to get a percentage value.

2.2.6.4 Statistical analysis

All statistical analyses were performed using the Graph Pad Prism 5 software. Statistical analysis was only performed when the sample size (n) was three or more.

When two conditions were compared, an unpaired Student´s t-test was used.

When more than two conditions were tested, a one-way ANOVA statistical analysis was performed. Either Tukey´s multiple comparison test of all columns or Dunnett's multiple comparison test against a control column was used as post test.

P-values < 0.05 were considered as significant.

3 Results

3.1 *Helicobacter pylori* interacts with Annexin A5

3.1.1 *Helicobacter pylori* P12 strain binds Annexin A5

The Annexin binding assay (see 2.2.1.7 and 3.1.3) was used to determine whether *H. pylori* is binding ANXA5. The GFP (green fluorescent protein)-expressing *H. pylori* strain P12 was incubated with fluorophore-coupled ANXA5. The binding of ANXA5 to the bacteria was then measured by observing the increase of the fluorescence in flow cytometry.

In the representative fluorescence histogram (see Figure 8A) an overlay of *H. pylori*-only (grey) and *H. pylori* with ANXA5 (red) can be seen. It shows that *H. pylori* binds ANXA5, as there is a clear shift of the curve towards the right showing an increase of the fluorescence due to the ANXA5 binding. Most of the measured bacteria bound ANXA5 and only some did not. With a quantitative analysis of the data in the FlowJo software (see 2.2.6.1) the percentage of fluorescence-positive events was determined (=ANXA5-binding bacteria) (see Figure 8B). Both P12 wildtype and P12 GFP (expressing cytoplasmic GFP) strains were tested and no difference could be observed between the two strains. Approximately ~85 % of the bacteria bound ANXA5.

The next step was to confirm that the events in the flow cytometer, which showed ANXA5 positivity, were actually the intact bacteria. Because GFP-expressing bacteria were used, this question could be assessed by analysing both fluorescences for each event (using dot plots of 488- (GFP signal) versus 647- (ANXA5 signal) channel). A representative dot plot can be seen in Figure 8C. The vast majority of bacteria showed a high 488-signal (quadrant 1 and 2), which indicates that the P12 strain was GFP-expressing as expected. With ANXA5 addition, most events, which showed a positive signal in the 647-channel, were also positive in the 488-channel (83.4 %, see quadrant 4). This observation suggests that the 647-positive (=ANXA5-positive) events are the intact bacteria, as they fluoresce also in green.

To further verify the *H. pylori*-ANXA5 interaction an additional biochemical method was used. The results are shown in Figure 8D. After the Annexin binding assay (with P12wt strain and fluorophore-coupled ANXA5) the samples were prepared and loaded to a single gel system polyacrylamide gel (see 2.2.4.1). ANXA5 alone was prepared and loaded as a positive control. In the Gel Doc documentation system the ANXA5 was made visible by excitation and detection of its coupled fluorophore. The data from this experiment reconfirm the flow cytometry data that *H. pylori* is binding ANXA5.

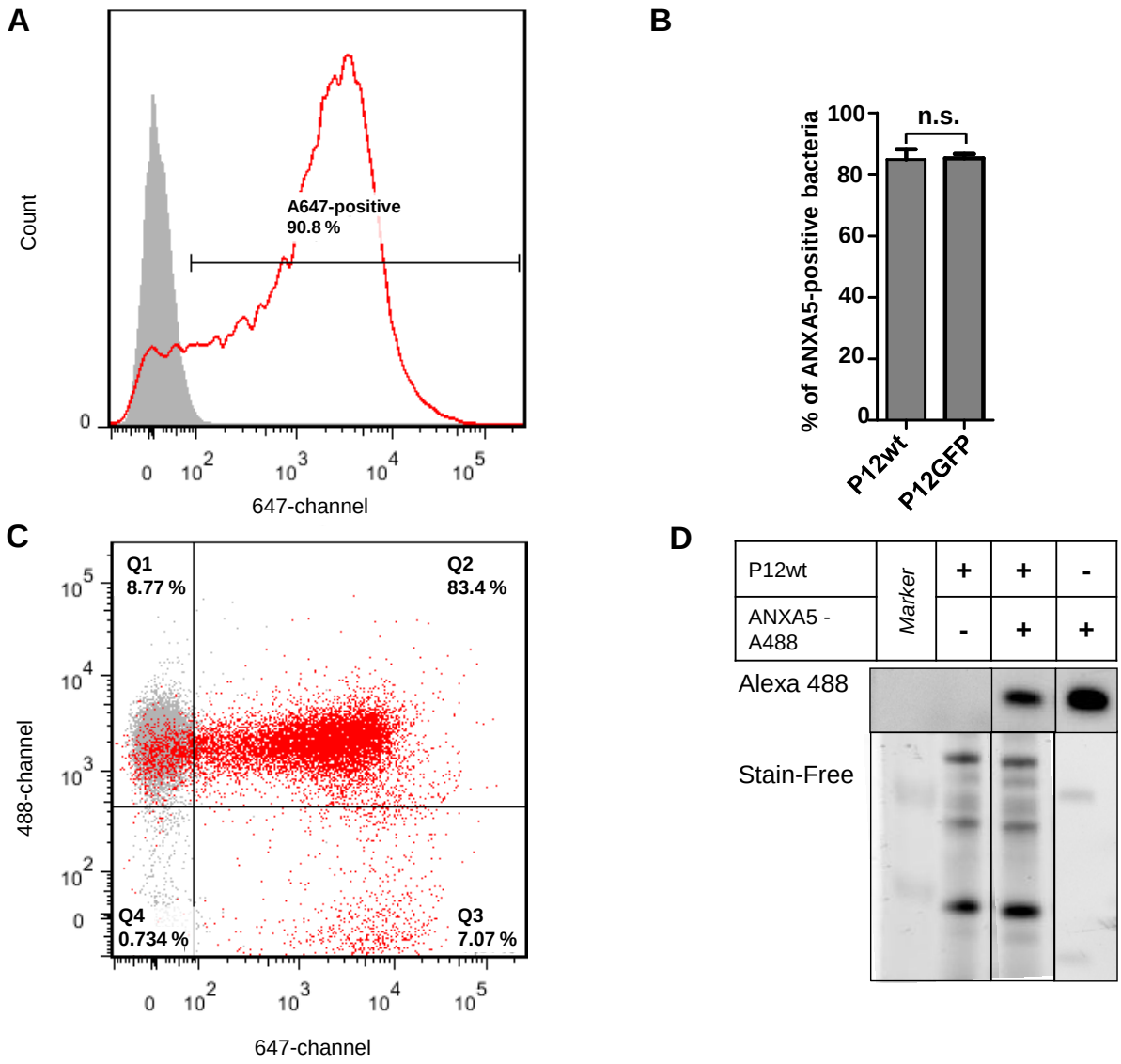


Figure 8 *Helicobacter pylori* is binding Annexin A5

The Annexin binding assay was performed using *H. pylori* strain P12 GFP and Alexa Fluor® 488- or 647-tagged ANXA5. Analyses were performed using flow cytometry (A-C) and gel electrophoresis (D).

(A) Representative histogram of the 647-fluorescence of *H. pylori* without (grey) and with (red) ANXA5 – Alexa 647 addition. For gating method see 2.2.6.1.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group were ~84.9 % for P12wt and ~85.2 % for P12 GFP; shown here with SEM. Unpaired t-test was performed: p=0.9284; n=4-5.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference
(C) GFP-expressing *H. pylori* was used. The dot plot shows 647- vs. 488-channel of *H. pylori* without (grey) and with (red) ANXA5-A647 addition. Quadrants are shown for the population with ANXA5 addition (red). Q2 and Q3 show the ANXA5-positive events; most of those events are also 488-positive (Q2).
(D) *H. pylori* and ANXA5-A488 were incubated, prepared and the samples were then loaded to a polyacrylamide gel. It was analysed in the gel documentation system by detecting the signal from the 488-fluorophore. ANXA5-A488 only was used as a positive control. The Stain-Free is shown as a control for the amount of protein loaded. n=3; a representative example is shown here. Experiment was performed by LJ.

Additionally, confocal laser scanning microscopy was done after performing the Annexin binding assay in order to visualize the *H. pylori*-ANXA5 interaction and to gain more information on where the bound ANXA5 was localized and how it was distributed.

An overview can be seen in Figure 9A. The *H. pylori* P12 strain used in this experiment expressed GFP and was stained with DAPI after fixation. Most bacteria showed a GFP signal (green), though the intensity of the signal varied. Only a few bacteria had already lost the GFP; those were most likely bacteria that had lost their membrane integrity, but still contained DNA and could therefore still be stained with DAPI (blue). Most bacteria seem to bind ANXA5 (red), which correlates with the flow cytometry data, though the amount of bound ANXA5 varied greatly. Whereas some bacteria bound little ANXA5 and only in one spot, others seemed to be completely “coated” in ANXA5. This impression can be seen in more detail in Figure 9B and 9C. While the *H. pylori* seen in Figure 9B only bound some ANXA5 at its very end, the *H. pylori* shown in the 3D reconstruction in Figure 9C bound a much higher amount of ANXA5 and is nearly completely surrounded by it.

For the first time it could therefore be shown, by using flow cytometry, gel electrophoresis as well as confocal laser scanning microscopy, that *H. pylori* is binding ANXA5.

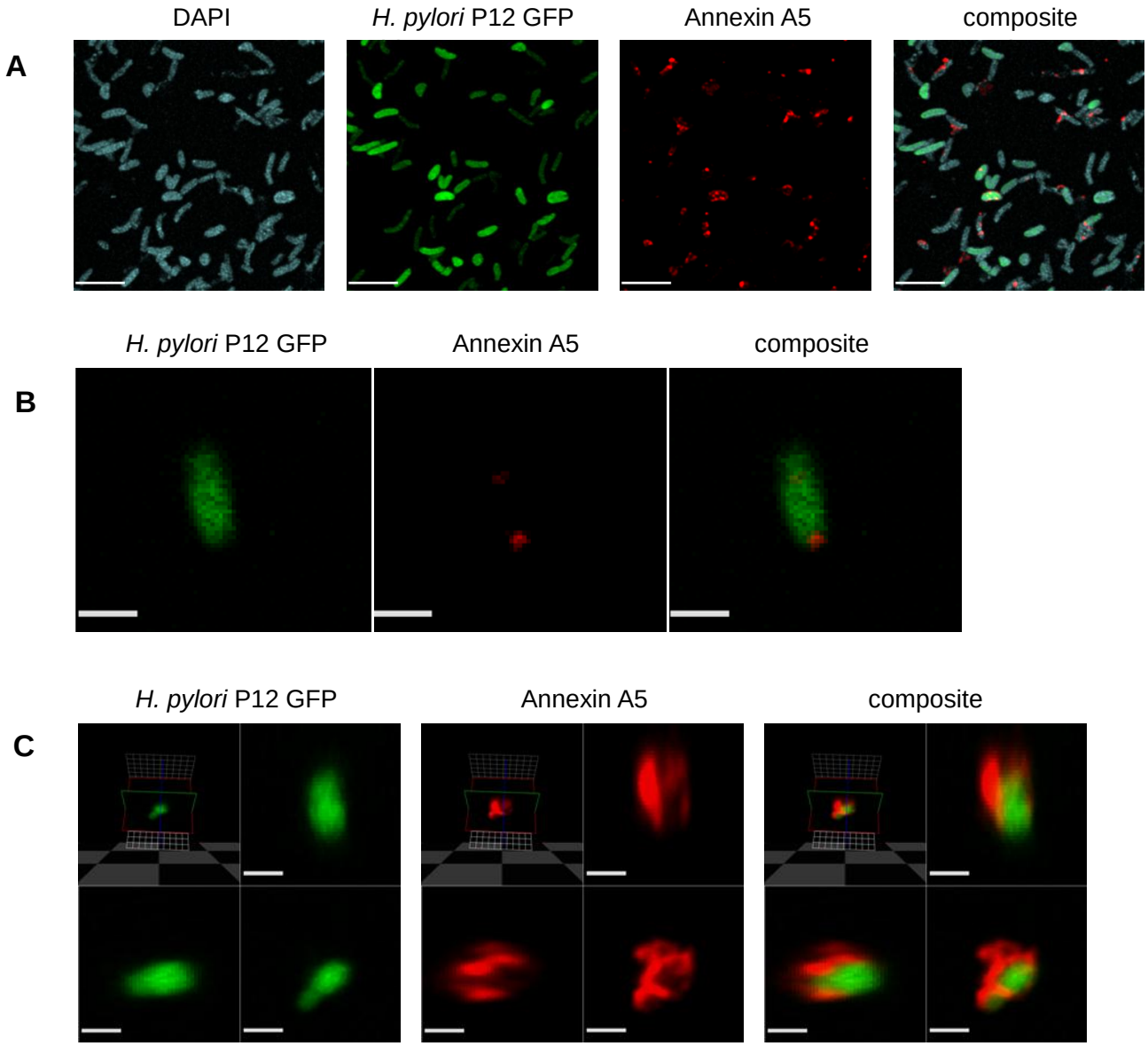


Figure 9 Confocal laser scanning microscopy of *H. pylori* binding Annexin A5

The Annexin binding assay was performed using *H. pylori* strain P12 GFP and Alexa Fluor® 594 or 647-tagged ANXA5. Analysis was performed using confocal laser scanning microscopy.

(A) Analysis with the confocal SP8 microscope and STED technology; GFP-expressing *H. pylori* P12 strain and ANXA5 Alexa 594 were used; the fixed samples were stained with DAPI. Scale bar 2.4 µm.
(B-C) Analysis with the confocal SP5 microscope and Volocity software; GFP-expressing *H. pylori* P12 strain and ANXA5 Alexa 647 were used. (C) shows a 3D reconstruction.
Scale bar equals 1 µm (B) and 1.3 µm (C).

3.1.2 Different *Helicobacter pylori* wildtype strains show different affinities to Annexin A5

Most experiments in this study were performed using the *H. pylori* wildtype strain P12. To investigate whether the binding of ANXA5 is a P12-specific effect, a variety of *H. pylori* wildtype strains were tested with the Annexin binding assay.

The analysis was performed by flow cytometry and both the percentage of bacteria binding ANXA5 and the amount of ANXA5 bound by the average of bacteria were looked at. The latter was determined with the median fluorescence intensity of the ANXA5-positive group of bacteria (see 2.2.6.1). Representative histograms of the data from flow cytometry are displayed in Figure 10.

The percentage of *H. pylori* that bound ANXA5 can be seen in Figure 11A. All tested *H. pylori* wildtype strains bound ANXA5, though the percentage of bacteria that bound varied. While nearly ~80 % of the bacteria from the P12 and *H. pylori* G27 strain bound ANXA5, only a quarter of bacteria from *H. pylori* Tx30a did. Tx30a strain showed significantly less binding compared to P12, while for all other strains the difference was not significant. The *H. pylori* 26695 and P217 strains were excluded from statistical analysis, because only two repeats had been performed on them. However, the preliminary data suggest that the ANXA5 binding of the 26695 strain was also reduced as compared to the P12 strain.

It was not only determined how many bacteria from each strain bound ANXA5, but also how much ANXA5 was bound by the average bacterium. To determine this, the median fluorescence intensity of the ANXA5-binding group was used (see 2.2.6.1). These data (see Figure 11B) show that the P12 strain bound higher amounts of ANXA5 than most other strains. The difference was significant for P145, X47 and Tx30a. The 26695 and P217 strains were once more excluded from statistical analysis, but again the preliminary data for 26695 seem to suggest that less ANXA5 is binding.

In addition to the analysis by flow cytometry, gel electrophoresis was performed with strains P12, 26695 and Tx30a (see Figure 11C). Therefore, the Annexin binding assay was performed with fluorophore-coupled ANXA5 and the prepared samples were loaded to a polyacrylamide gel (see 2.2.1.7.4). The fluorophore was excited and recorded with the gel documentation system**.** This assay confirmed that all three tested strains bound ANXA5. However, this method does not show a difference between the amounts of ANXA5 bound by the different wildtype strains.

These experiments show that the ANXA5 binding is not unique for the P12 strain, but prevalent in all tested *H. pylori* wildtype strains. The ANXA5-binding capacity varies, though, with the P12 strain being one of the strains with the highest ANXA5-binding capacity.

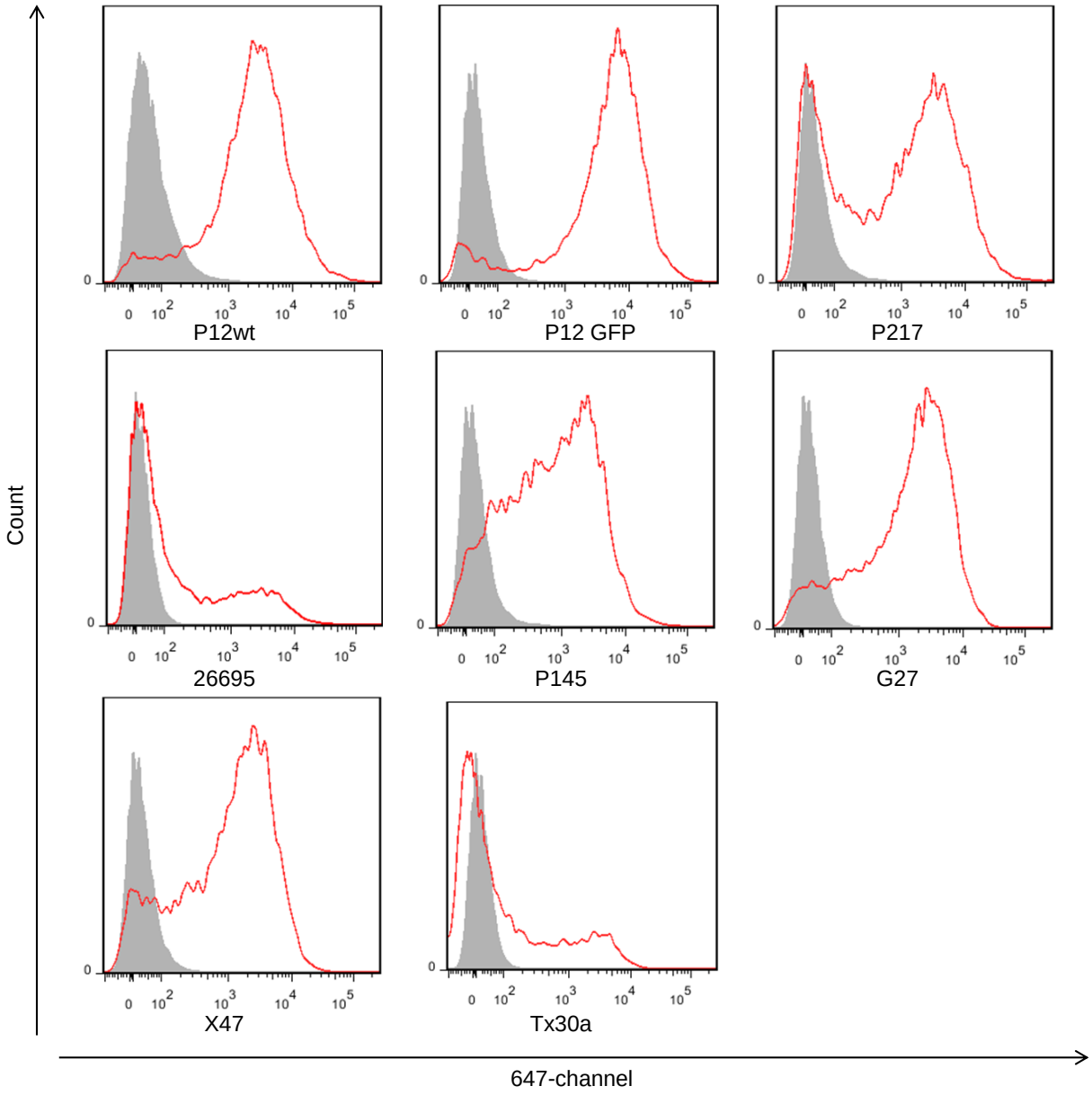


Figure 10 Different wildtype strains of *Helicobacter pylori* binding Annexin A5 (1)

The Annexin binding assay was performed using different *H. pylori* wildtype strains and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.

Representative histograms of the 647-fluorescence of the *H. pylori* wildtype strains without (grey) and with (red) ANXA5-A647 addition.

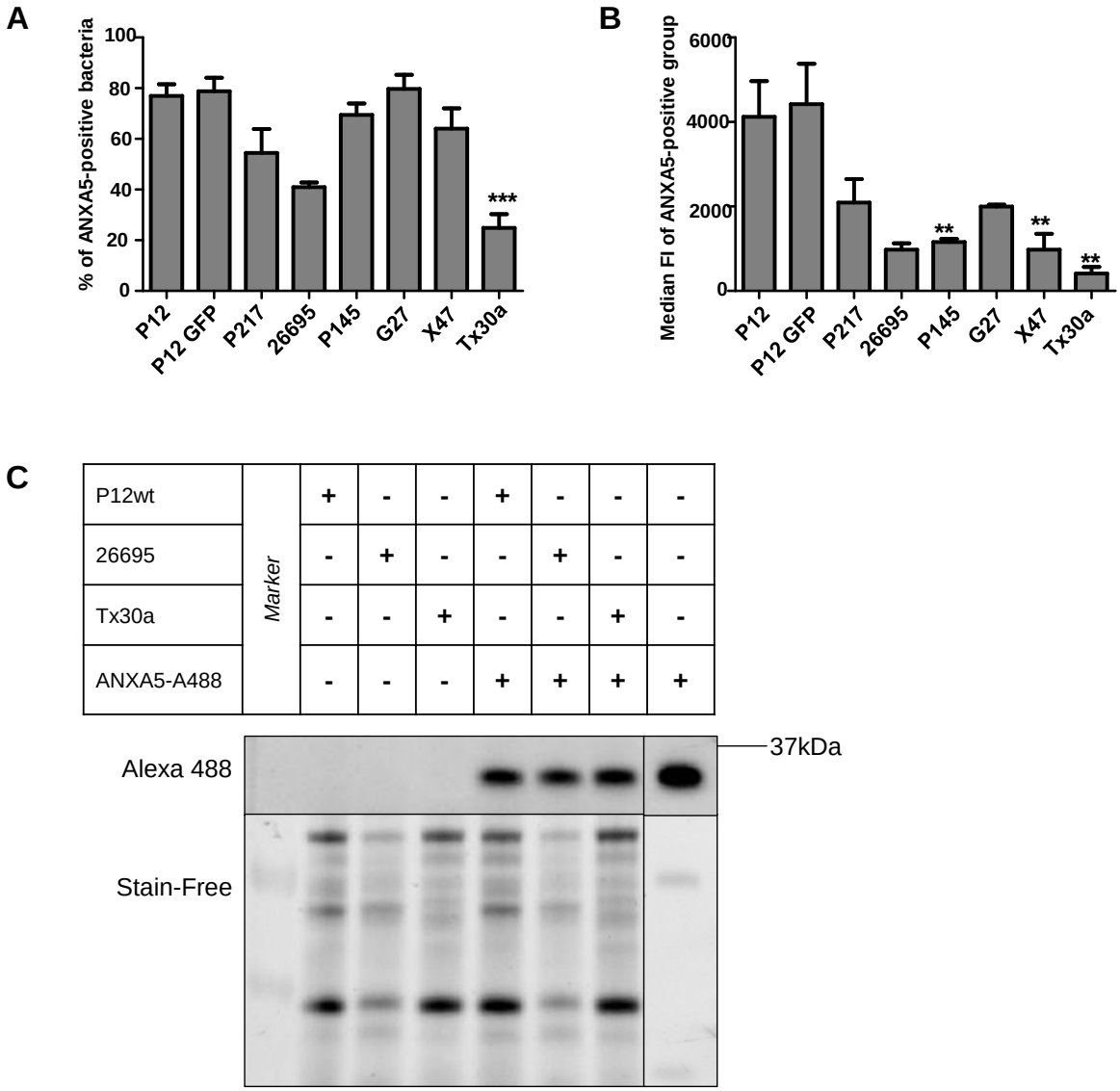


Figure 11 Different wildtype strains of *Helicobacter pylori* binding Annexin A5 (2)

The Annexin binding assay was performed using different *H. pylori* wildtype strains and Alexa Fluor® 647-(A, B) or 488-(C) tagged ANXA5. Analyses were performed using flow cytometry (A, B) or gel electrophoresis (C).

(A) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group is shown here with SEM. One-way ANOVA ($p<0.0001$) and Dunnett's multiple comparison post-test against P12wt as control were performed and showed a high significance for Tx30a strain; n=3; P217 and 26695 strain were excluded from statistical analysis, as n=2.
(B) The graph shows the mean value with SEM of the median 647-fluorescence intensity of the gated "ANXA5-positive group" (see (A)). One-way ANOVA ($p=0.0006$) and Dunnett's multiple comparison post-test against P12wt as control were performed and showed a high significance for P145, X47 and Tx30a strains. n=3; P217 and 26695 strain were excluded from statistical analysis, as n=2.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$, n.s. = no significant difference
(C) *H. pylori* strains P12wt, 26695 and Tx30a were incubated with ANXA5-A488; the samples were then prepared and loaded to a polyacrylamide gel. It was analysed in the gel documentation system by detecting the signal from the 488-fluorophore. ANXA5-A488 only was used as a positive control. The Stain-Free is shown as a control for the amount of protein loaded. n=3; a representative example is shown here. Experiment was performed by LJ.

3.1.3 Standardization of the Annexin binding assay

H. pylori strains bind ANXA5 (see 3.1.1). The Annexin binding assay (see 2.2.1.7) was newly established for this study. Therefore, it was important to know the conditions in which ANXA5 binds to bacteria by standardizing the assay. The conditions tested included varying amounts of ANXA5, varying amounts of bacteria, different incubation media and different incubation temperatures.

3.1.3.1 Slight effect of different incubation media on the Annexin A5 binding of Helicobacter pylori

It has been shown that the binding of ANXA5 to phospholipids depends on various aspects, for example the calcium concentration.[149] It was therefore crucial to define the binding capacity of ANXA5 to *H. pylori* in different incubation media in comparison to the standard Annexin A5 binding buffer (AnxBuf). The assay was hence performed in either RPMI, complete medium (CM) or AnxBuf (see 2.1.4).

Figure 12A shows representative histograms of the fluorescence of bacteria that have been incubated with fluorophore-coupled ANXA5 (red curve) in the different media. While the curves for RPMI and AnxBuf look quite similar, the curve for CM is shifted to the left. This means the bacteria show a lower ANXA5 binding, when being incubated in CM.

This impression is confirmed by the quantitative analysis (see Figure 12B). In RPMI and AnxBuf over ~85 % of bacteria bound ANXA5, whereas only approximately ~66 % did so, when they were incubated in CM. The ANXA5 binding was not significantly different whether incubation was performed in AnxBuf or RPMI.

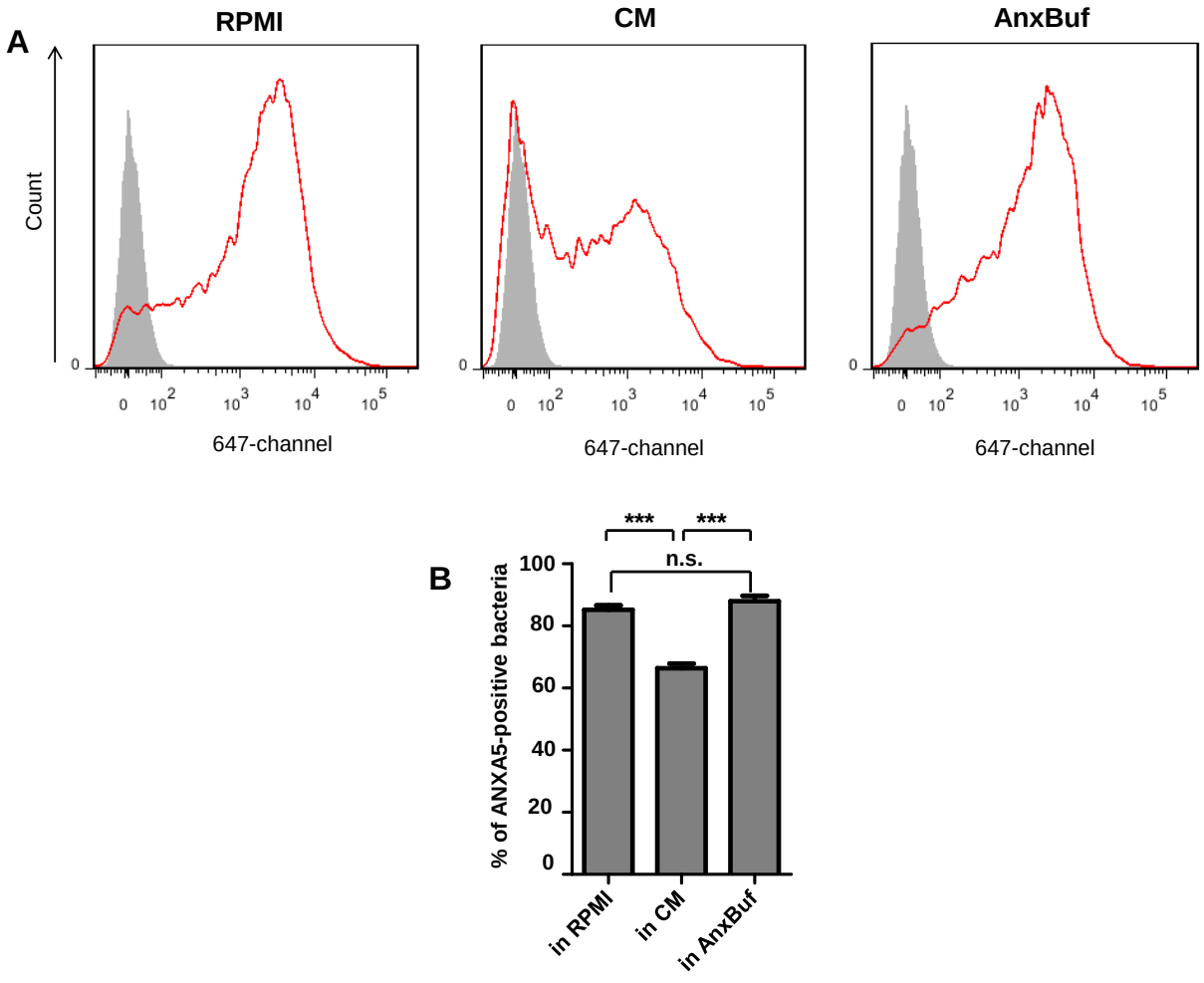


Figure 12 Role of incubation medium for *Helicobacter pylori* binding Annexin A5

The Annexin binding assay was performed using *H. pylori* P12 GFP strain and Alexa Fluor® 647-tagged ANXA5 and was incubated in either RPMI, complete medium (CM) (= RPMI media complemented with foetal calf serum) or Annexin A5 binding buffer (AnxBuf). Analysis was performed using flow cytometry.
(A) Representative histograms of the 647-fluorescence of the bacteria in RPMI, CM or AnxBuf (from left to right). *H. pylori* only (grey) and with ANXA5 addition (red) is displayed.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are displayed here +/- SEM. One-way ANOVA ($p<0.0001$) and Tukey's multiple comparison post-test was performed; n=4.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$, n.s. = no significant difference

3.1.3.2 Annexin A5 binding is slightly reduced after incubation at 4°C compared to 37°C

It was necessary to test whether the incubation temperature has any effect on the ANXA5 binding, as annexins bind to phospholipids integrated into cellular membranes and their properties, like membrane fluidity, depend amongst other things on the temperature.[69,150] Therefore, the incubation of the assay was performed at either 4°C or 37°C. The results can be seen in Figure 13. When *H. pylori* was incubated with ANXA5 at 4°C, fewer bacteria

bound ANXA5 than at 37°C. This could be observed in RPMI as well as in Annexin A5 binding buffer (see Figure 13A and B, respectively).

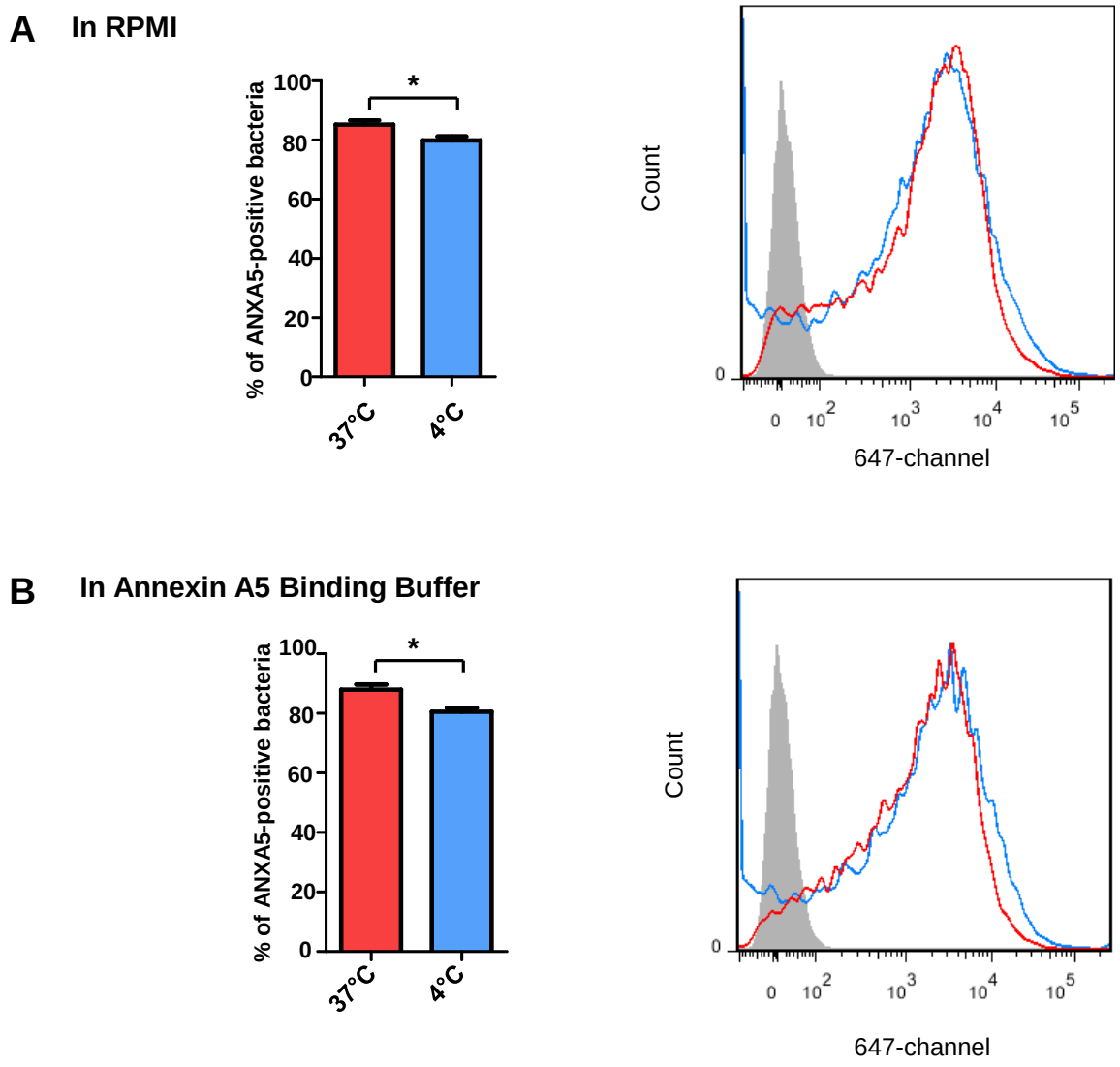


Figure 13 Role of incubation temperature for *Helicobacter pylori* binding Annexin A5

The Annexin binding assay was performed using *H. pylori* P12 GFP strain and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.
The assay was incubated in either Annexin A5 binding buffer (A) or RPMI (B) at 37°C (red) or 4°C (blue).
Left: With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are displayed here +/- SEM. Unpaired t-tests were performed and showed a significant difference ((A): p=0.0356; (B): p=0.0133); n=4.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference
Right: Furthermore, representative histograms of the 647-fluorescence of the bacteria with incubation with ANXA5 at 37°C (red) or 4°C (blue) are shown. *H. pylori* only (without ANXA5) is displayed in grey.

3.1.3.3 Effect of varying amount of bacteria/ Annexin A5

Further tests were conducted to examine whether another ratio between *H. pylori* and ANXA5 has any effect on the binding capacity and if there is a saturation effect. Experiments were performed with a varying OD_{550} of bacteria and with varying amounts of ANXA5. The aim was to find the ideal ratio for future experiments.

First, a varying amount of bacteria was tested in the Annexin binding assay. In Figure 14 the results of this experiment can be seen. It appears that the higher the number of bacteria, the lower the binding of ANXA5. There was always the same amount of ANXA5 in each tube and therefore it seems quite logical, that the lower ODs show a higher binding signal (as there simply is more ANXA5 per bacterium available). This trend was not statistically significant, though, as the variation was quite high. The proportion between the different ODs was the same in all replicates, but the absolute values showed a high variation between the different repeats of the experiment.

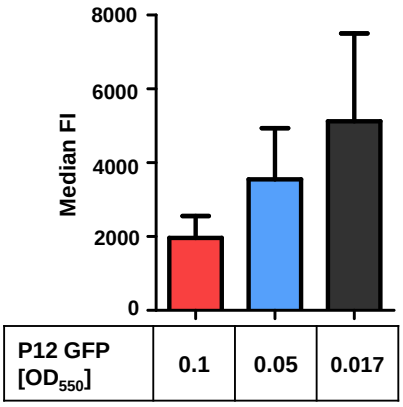


Figure 14 Influence of the amount of *Helicobacter pylori* on Annexin A5 binding (1)

The Annexin binding assay was performed with different amounts of P12 GFP (OD_{550} 0.1, 0.05 and 0.017) and ANXA5-Alexa Fluor® 647 (1.5 µl).
Median fluorescence intensity from the 647-channel is displayed with SEM. One-way ANOVA was performed, p=0.4416.
* p<0.05; ** p<0.01; *** p<0.001

To determine which effect different ratios between *H. pylori* and ANXA5 would have on the binding capacity, not only varying amounts of bacteria, but also varying amounts of ANXA5 were tested. In Figure 15A a representative histogram of this experiment can be seen. It shows that the curve with addition of higher amounts of ANXA5 (shown in blue) is shifted to the right as compared to the one with only low amounts of ANXA5 (shown in red). This means that more ANXA5 was bound, when more ANXA5 was added.

The quantitative analysis (see 2.2.6.1) shows that there is no difference in the percentage of bacteria binding ANXA5 (see Figure 15B), but that the median fluorescence intensity (MFI)

was higher, when more ANXA5 was added (Figure 15C; MFI was ~9.500 as compared to ~2.500; also see 2.2.6.1). This means that there were not more bacteria that bound ANXA5, when more ANXA5 was added (as even with low amounts of ANXA5 the percentage is already quite high (>80 %)). However, those bacteria that bound ANXA5 harboured significantly more in average.

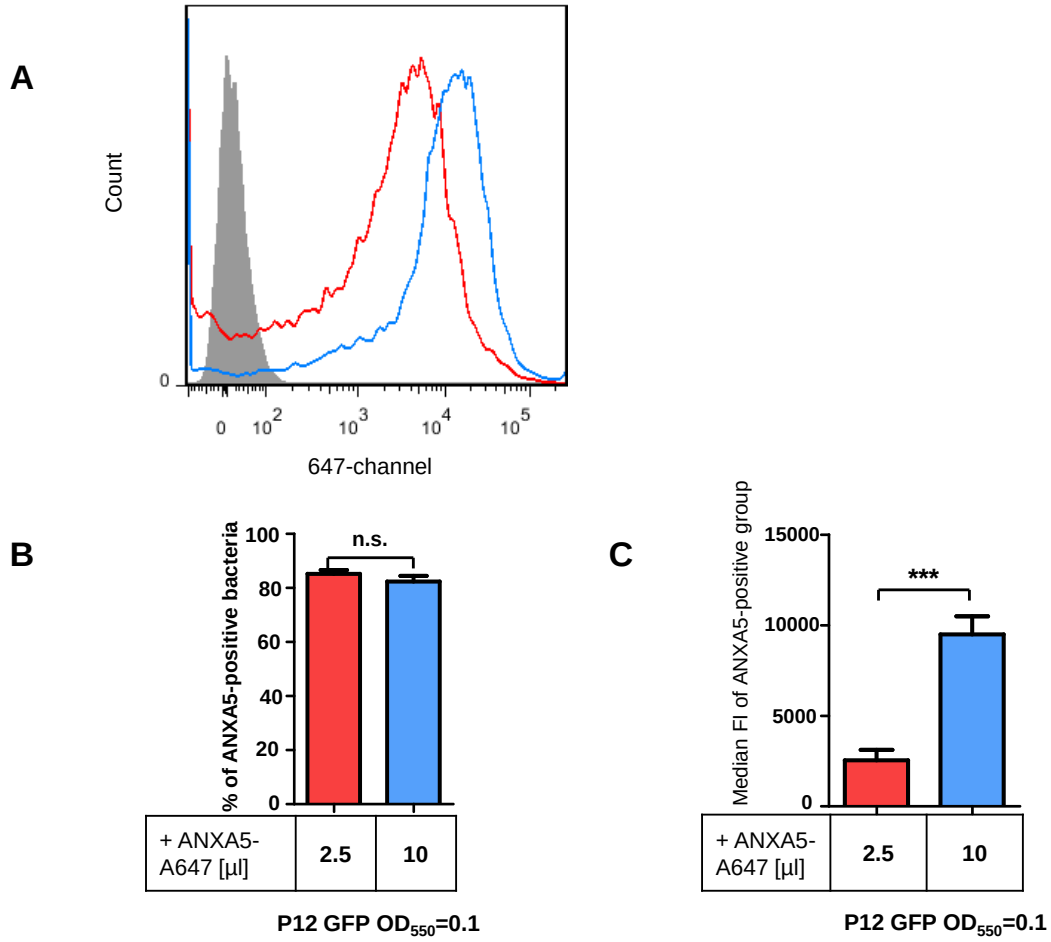


Figure 15 Different amounts of Annexin A5

The Annexin binding assay was performed with P12 GFP and different amounts of ANXA5-Alexa Fluor® 647 (2.5 and 10 µl) and was measured in the flow cytometer.

(A) Representative histogram with P12 only (grey) and with 2.5 µl (red) or 10 µl (blue) ANXA5-A647.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are shown here with SEM. Unpaired t-test was used: p=0.3014.
(C) The graph shows the mean value with SEM of the median 647-fluorescence intensity of the gated "ANXA5-positive group" (see (B)). Unpaired t-test was used: p=0.0009.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

To complete these investigations and to further identify and confirm the optimal amount of bacteria for future experiments the different amounts of bacteria were also tested with a higher amount of ANXA5. Figure 16A shows a representative histogram. The curves between the three different ODs are congruent, which means that the ANXA5 binding is

nearly the same. The quantitative analysis (see Figure 16B) also supports these data. There is no significant difference in the ANXA5-binding capacity between the different ODs.

While there was a difference (or at least a trend) between the ODs when only small amounts of ANXA5 were added (see Figure 14), there is no difference when high amounts of ANXA5 were added. This suggests that a saturation effect takes place and that the binding capacity of the bacteria has reached its limit.

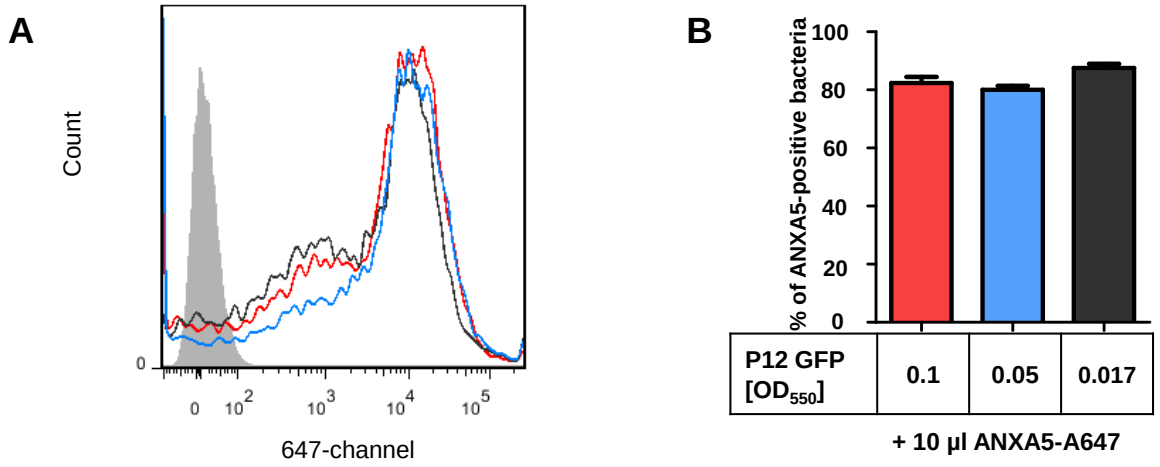


Figure 16 Influence of the amount of *Helicobacter pylori* on Annexin A5 binding (2)

The Annexin binding assay was performed with different amounts of P12 GFP (OD_{550}= 0.1, 0.05 and 0.017) and ANXA5-Alexa Fluor® 647 (10 µl).

(A) Representative histogram with P12 GFP only (grey) and with 10 µl of ANXA5-A647 in OD_{550}=0.1 (red), 0.05 (blue) and 0.017 (black).
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are shown here with SEM. One-way ANOVA was performed, p=0.0618.
* p<0.05; ** p<0.01; *** p<0.001

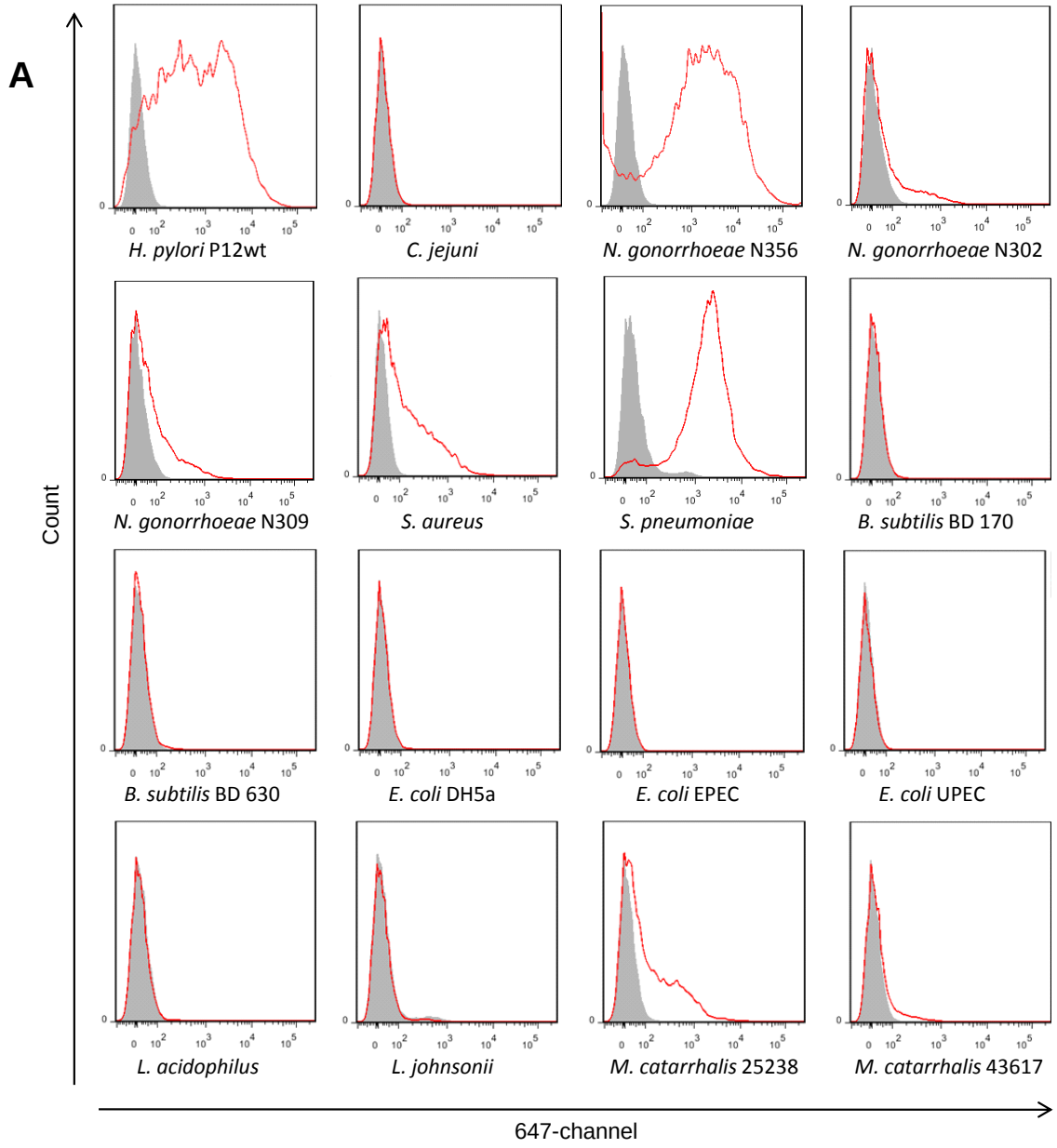
3.2 Annexin A5 binds to *Helicobacter pylori* and few other bacteria

To investigate how specific the ANXA5 interaction is for *H. pylori,* a variety of other bacteria were additionally tested. This was especially important, as it has been shown that some other bacteria and viruses also interact with annexins (see 1.2.4). 16 different, gram-positive and gram-negative, aerobic and microaerophilic as well as human pathogenic and non-pathogenic bacterial strains were used in this study.

The Annexin binding assay (see 2.2.1.7) with fluorophore-coupled ANXA5 was performed with all strains, the samples were measured in the flow cytometer and the data were analysed with the FlowJo software.

Figure 17A shows representative fluorescence histograms showing the ANXA5 binding. In Figure 17B the percentage of bacteria that bound ANXA5 is displayed. The bacterium *Campylobacter jejuni* was of special interest, as it is a very close relative to *H. pylori* and did surprisingly not bind ANXA5.[2] Therefore, *H. pylori* strain P12wt was used as positive and *C. jejuni* as negative control. Statistical analysis was performed and all bacteria were compared with both, the positive and the negative control (see Table 3). Less than half of the tested bacterial species showed a significant difference to *C. jejuni*, which means that only those bacteria did bind ANXA5. All strains except the *Neisseria gonorrhoeae* strain N356 showed a significant difference to *H. pylori* as the positive control. This leads to the conclusion that they bound significantly less ANXA5 when compared to *H. pylori*, or did not bind at all.

In Table 3 an overview of how the different bacterial strains behaved as compared to the controls and whether they bound ANXA5 or not can be seen. (For further analysis of the results see 4.1.)


A
Count
H. pylori P12wt
C. jejuni
N. gonorrhoeae N356
N. gonorrhoeae N302
N. gonorrhoeae N309
S. aureus
S. pneumoniae
B. subtilis BD 170
B. subtilis BD 630
E. coli DH5a
E. coli EPEC
E. coli UPEC
L. acidophilus
L. johnsonii
M. catarrhalis 25238
M. catarrhalis 43617
647-channel

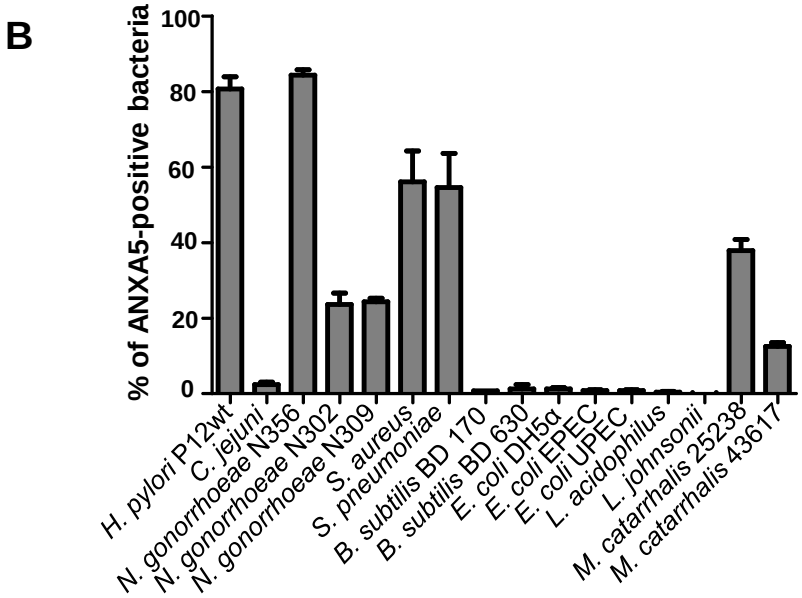

B
% of ANXA5-positive bacteria
100
80
60
40
20
0
H. pylori P12wt
C. jejuni
N. gonorrhoeae N356
N. gonorrhoeae N302
N. gonorrhoeae N309
S. aureus
S. pneumoniae
B. subtilis BD 170
B. subtilis BD 630
E. coli DH5a
E. coli EPEC
E. coli UPEC
L. acidophilus
L. johnsonii
M. catarrhalis 25238
M. catarrhalis 43617

Figure 17 Different bacteria binding Annexin A5

The Annexin binding assay was performed using different bacteria and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.

(A) Representative histograms of the 647-fluorescence of the different bacteria without (grey) and with (red) ANXA5-A647 addition. The y-axis shows the count and the x-axis the 647-channel fluorescence intensity.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group is shown here with SEM. For statistical analysis see Table 3 below.

Table 3 Different bacteria binding Annexin A5

See Figure 17B above. Statistical analysis:
One-way ANOVA ($p<0.0001$) was performed. Subsequently, Dunnett's multiple comparison post-test was performed against the control columns *H. pylori* P12wt (as positive control for ANXA5 binding) and *C. jejuni* (as negative control for ANXA5 binding).
All bacteria except *N. gonorrhoeae* N356 showed a highly significant reduction of ANXA5 binding when compared to P12wt.
Only *N. gonorrhoeae* (N356, N302 and N309), *S. aureus, S. pneumoniae* and *M. catarrhalis* (25238) showed a significant to very significant difference to the negative control *C. jejuni* and therefore bind ANXA5.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$, n.s. = no significant difference

	Compared to control:		**ANXA5 binding?**
	Positive *(H. pylori* P12wt*)*	**Negative *(C. jejuni)***	
***N. gonorrhoeae* N356**	n.s.	***	**yes**
***N. gonorrhoeae* N302**	***	*	**yes**
***N. gonorrhoeae* N309**	***	*	**yes**
S. aureus	***	***	**yes**
S. pneumoniae	***	***	**yes**
***B. subtilis* BD 170**	***	n.s.	no
***B. subtilis* BD 630**	***	n.s.	no
***E. coli* DH5α**	***	n.s.	no
***E. coli* EPEC**	***	n.s.	no
***E. coli* UPEC**	***	n.s.	no
L. acidophilus	***	n.s.	no
L. johnsonii	***	n.s.	no
***M. catarrhalis* 25238**	***	***	**yes**
***M. catarrhalis* 43617**	***	n.s.	no

3.3 The Annexin A5–*Helicobacter pylori* interaction seems to be lipid- rather than protein-mediated

Annexins (ANXs) can interact with membranes in three different ways: i) by calcium-dependent binding of their C-terminal domain to lipids, ii) by calcium-independent binding mediated by lipids and iii) directly via the N-terminal domain to membrane proteins (see 1.2.1 and Figure 33). In order to determine the nature of the ANXA5 interaction with *H. pylori* these three options were evaluated.

3.3.1 The Annexin A5 binding of *Helicobacter pylori* is calcium-dependent

To get a better understanding of the *H. pylori*-ANXA5 interaction it was interesting to investigate whether the binding was dependent on the presence of calcium ions or not, as the most commonly known form of interaction between ANXs and membrane phospholipids is calcium-dependent (see 1.2.1). Hence, the Annexin binding assay was performed with the chelator EGTA, which is able to bind divalent ions and especially shows a high affinity to calcium ions. EGTA should therefore reduce the level of free calcium in the medium to a minimum.

The Annexin binding assay was performed in Annexin A5 binding buffer (AnxBuf) and RPMI, with or without EGTA (for normal calcium concentrations of these media see Table 4). The samples were measured in the flow cytometer and were analysed using the FlowJo software. The percentage of bacteria that bound ANXA5 was examined as well as the amount of ANXA5 bound by the average of the bacteria. The latter was determined with the median fluorescence intensity of the ANXA5-positive group of bacteria (as fluorophore-coupled ANXA5 was used) (see 2.2.6.1).

Table 4 Calcium concentration of media used for Annexin binding assay

Calcium concentration	[mM]
Annexin A5 binding buffer	1.8
RPMI	0.42

Figure 18 shows the results for both AnxBuf (A) and RPMI (B). When EGTA was added (blue), significantly fewer bacteria bound ANXA5 (from over ~85 % without EGTA to ~8 % (AnxBuf)/ ~15 % (RPMI)). Moreover, the median fluorescence intensity (MFI) decreased,

which means that those *H. pylori* that still bound ANXA5 after EGTA addition bound significantly lower amounts of ANXA5 (hardly any). Based on these results it can be concluded that the *H. pylori*-ANXA5 interaction is calcium-dependent.

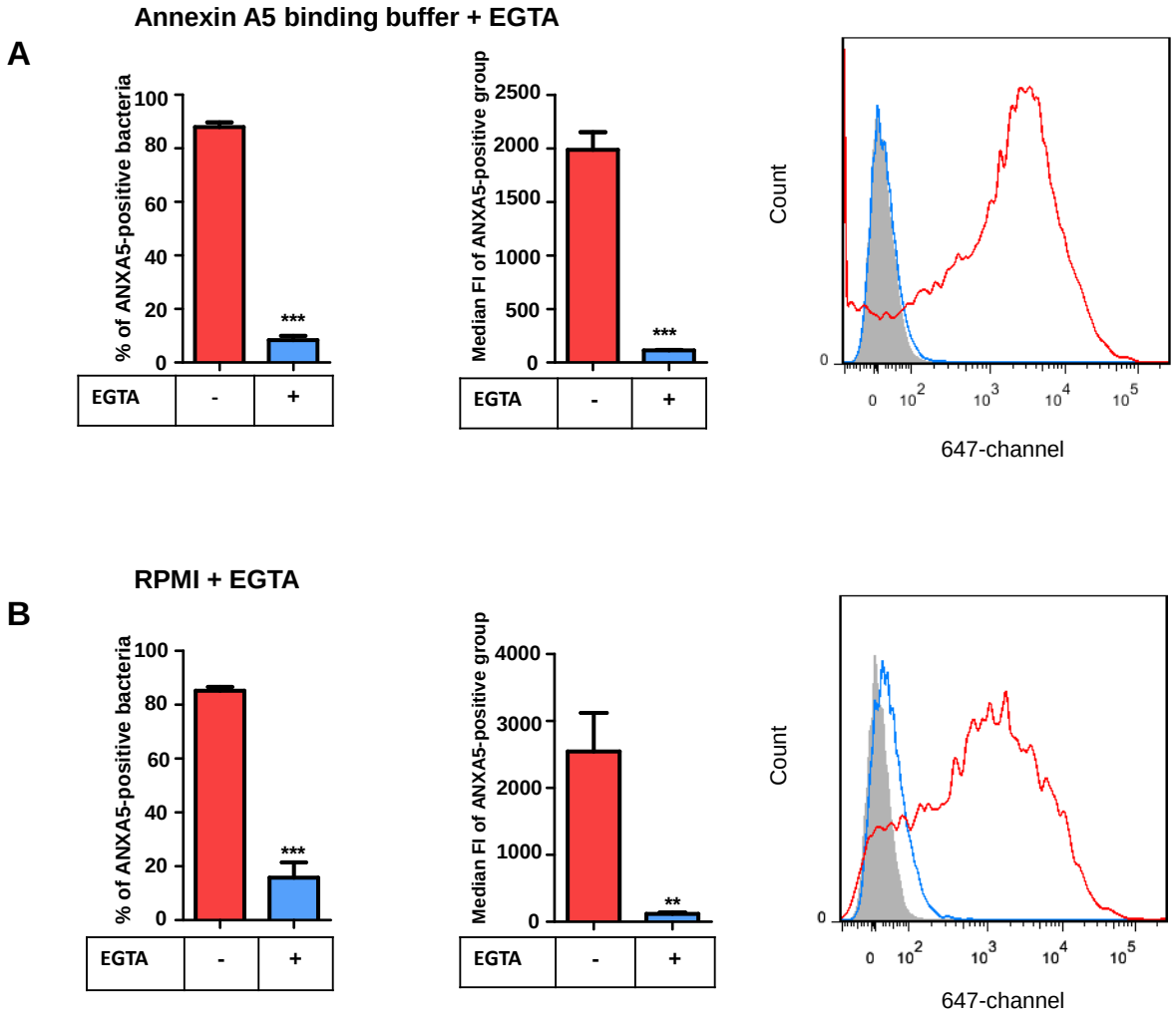


Figure 18 Calcium dependency of *Helicobacter pylori* binding Annexin A5 (1)

The Annexin binding assay was performed using *H. pylori* P12 GFP strain and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.
(A-B) The assay was incubated in Annexin A5 binding buffer (A) and RPMI (B) with (blue) and without (red) EGTA 5mM.
With FlowJo the flow cytometry data were gated to the 647-positive events; the mean percentage of bacteria in this group is shown in the left graph +/- SEM. The mean +/- SEM of the median fluorescence intensity (MFI) is shown in the right graph. Unpaired t-tests were performed and showed a highly ($p=0.0056$) to very highly ($p<0.0001$) significant difference; n=4.
Furthermore, a representative histogram of the 647-fluorescence of the bacteria with (blue) and without (red) EGTA is shown. In grey *H. pylori* only (without ANXA5) is displayed.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

Additionally, it was tested whether an increased calcium concentration has any effect on the ANXA5 binding. Therefore $CaCl_2$ was added to RPMI, so that RPMI had the same calcium

concentration as the AnxBuf, as this buffer was specifically designed to investigate ANX interactions (see Table 4).[138]

When $CaCl_2$ was added in the Annexin binding assay, a shift of the fluorescence histogram to the right (which indicates higher ANXA5 binding) could be observed (see Figure 19, right graph). More *H. pylori* bound ANXA5 (~93 % compared to ~85 %) and the bacteria that bound ANXA5 bound higher amounts on average (which can be seen by the increase of the MFI) (see Figure 19, left and middle graph, respectively). When more calcium is present, more bacteria bound ANXA5 and they also bound higher amounts of ANXA5. These findings strongly indicate that calcium plays an essential role in the ANXA5-*H. pylori* interaction.

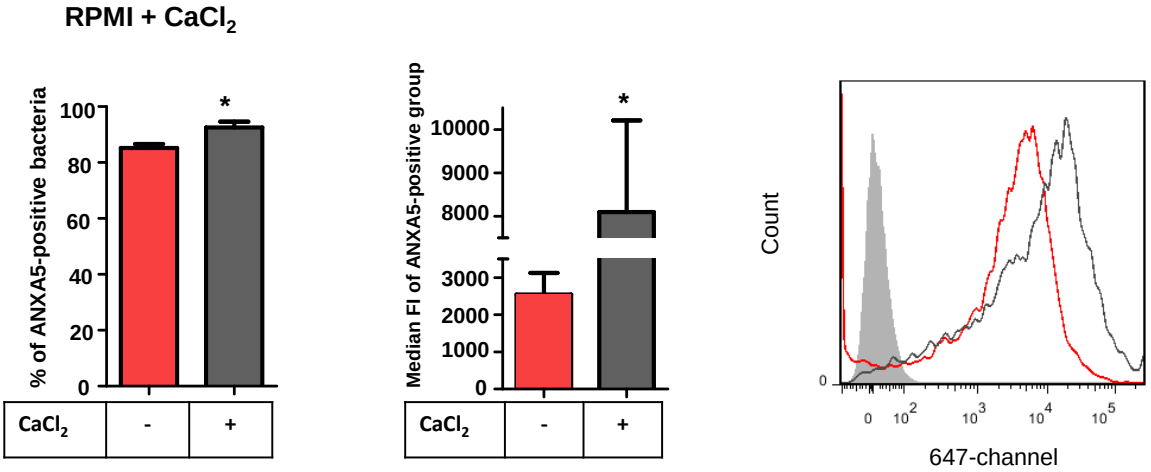


Figure 19 Calcium dependency of *Helicobacter pylori* binding Annexin A5 (2)

The Annexin binding assay was performed using *H. pylori* P12 GFP strain and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.
The assay was incubated in RPMI without (red) and with (dark grey) $CaCl_2$ (final Ca^{2+}-concentration: 0.42 and 1.8 mM, respectively). With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group is shown in the left graph +/- SEM. The mean +/- SEM of the median fluorescence intensity (MFI) is shown in the right graph. Unpaired t-tests were performed and showed a significant difference (p=0.0288 for the left and p=0.0444 for the right graph); n=4.
Furthermore, a representative histogram of the 647-fluorescence of the bacteria with (dark grey) and without (red) $CaCl_2$ is shown. *H. pylori* only (without ANXA5) is displayed in grey.
* p<0.05; ** p<0.01; *** p<0.001

3.3.2 Denaturation of proteins does not affect *Helicobacter pylori´s* binding of Annexin A5

The findings from 3.3.1 show that the ANXA5 binding is calcium-dependent and therefore suggest that the binding might be lipid- rather than membrane protein-mediated. *H. pylori* should hence still bind ANXA5 when its proteins have been destroyed. To test this hypothesis, protein denaturation was performed by exposing *H. pylori* for 5 minutes to different temperatures (as different proteins show denaturation at different temperatures). After this, the Annexin binding assay was performed and the results were measured by flow cytometry.

The bacteria, which had undergone protein denaturation before, still bound ANXA5 and even showed a slightly higher ANXA5 binding. This can be observed in the representative histogram in Figure 20A, where their curve is slightly shifted to the right as compared to the non-boiled control (shown in grey). The quantitative analysis also shows a significant difference as compared to the control. While ~85 % of the bacteria bound ANXA5, when they were not boiled before, the percentage of binding bacteria increased to approximately ~95 %, when the proteins were denatured (Figure 20B).

The success of the protein denaturation treatment was validated by checking the function of the green fluorescent protein (GFP). The *H. pylori* strain used was GFP-expressing and showed a positive GFP signal, whereas the temperature-treated bacteria did not show any GFP signal (see representative histogram, Figure 20C). Consequently, it can be concluded that all temperature-based treatments used for denaturation of proteins were successful.

Taken together, these data showed that the ANXA5 binding of *H. pylori* is not dependent on the integrity of *H. pylori´s* proteins and is therefore most likely lipid-mediated, which supports the results from chapter 3.3.1.

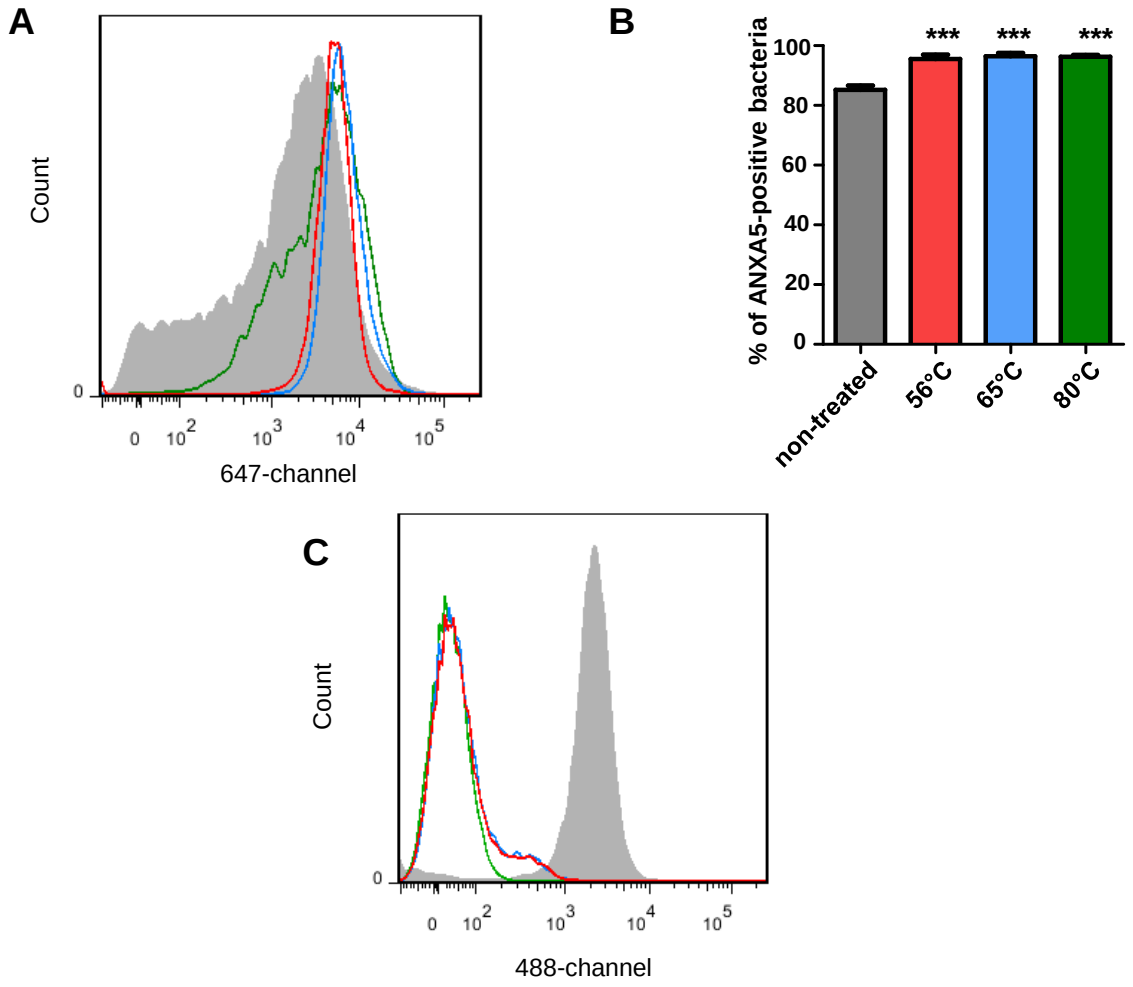


Figure 20 ANXA5 binding still takes place after protein denaturation

The Annexin binding assay was performed using *H. pylori* P12 GFP strain, which had been heated to 56°C, 65°C or 80°C, before Alexa Fluor® 647-tagged ANXA5 was added. Analysis was performed using flow cytometry.
(A) Representative overlay histogram of the 647-fluorescence of the bacteria, that had been treated at 56°C (red), 65°C (blue) or 80°C (green) with ANXA5. *H. pylori* that had not been temperature treated is shown in grey.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are displayed here +/- SEM. Non-treated bacteria (grey) were compared to bacteria with protein denaturation at 56°C, 65°C or 80°C (displayed in red, blue and green, respectively). Statistical analysis was done with one-way ANOVA ($p<0.0001$) and Dunnett's multiple comparison post-test against the non-treated control; n=3-4.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$
(C) Representative histogram of the 488-fluorescence of the bacteria. This was used as a control that the protein denaturation worked, as the GFP protein was destroyed. Same colour code used as in (A).

3.3.3 Blocking LPS with Polymyxin B does not affect the Annexin A5 binding of *Helicobacter pylori*

H. pylori is a gram-negative bacterium and thus its membrane contains LPS, though its structure is quite unique (see 1.1.6). An interaction between certain bacteria and ANXA5 has been previously described and it was stated that the ANXA5 binding takes place via the LPS/ lipid A of the bacteria.[114] Based on these previous observations it was tested whether the ANXA5 binding of *H. pylori* still takes place when its LPS is blocked. Polymyxin B, a known LPS inhibitor, was used for this purpose.

The Annexin binding assay was performed and the bacteria were incubated with Polymyxin B before the assay. To ensure that not the incubation per se was relevant for any effects, a control was performed, where bacteria were just incubated in medium.

The blocking of LPS with Polymyxin B did not seem to make a difference in the ability of *H. pylori* to bind ANXA5 (see Figure 21) nor did the incubation as such (see 4.1 for further discussion).

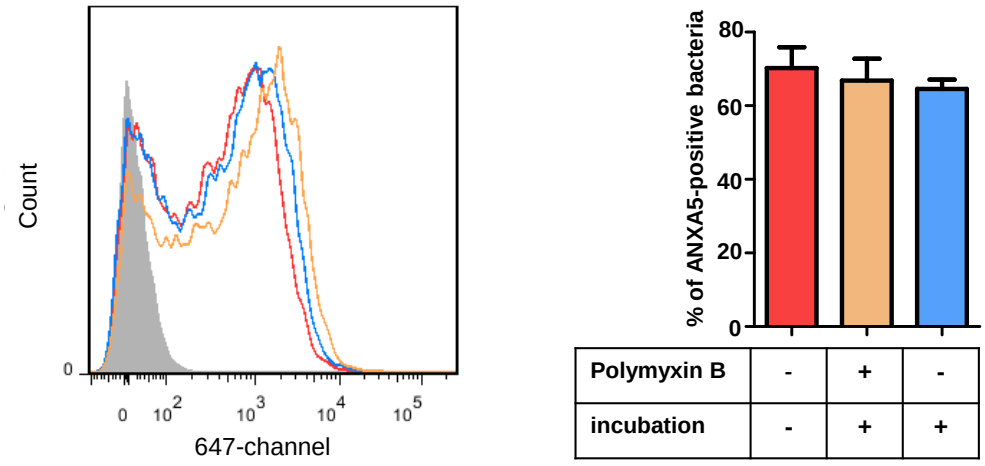


Figure 21 Role of LPS for the *Helicobacter pylori*-Annexin A5 interaction

The Annexin binding assay was performed using *H. pylori* P12 GFP strain and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.
Bacteria were partly pre-incubated with Polymyxin B (blocking LPS) or pre-incubated only as a control.
A representative histogram of the 647-fluorescence of the bacteria with Polymyxin B incubation (yellow), incubation only (blue) and without both (red) is shown on the left. In grey *H. pylori* only (without ANXA5) is displayed.
With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group is shown in the right graph +/- SEM.
One-way ANOVA was performed and showed no significant difference (p=0.7302); n=3.
* p<0.05; ** p<0.01; *** p<0.001

3.4 Cholesterol metabolism does not alter the *Helicobacter pylori*-Annexin A5 interaction

It has been previously published that *H. pylori* can extract cholesterol from its growth medium and integrate the glycosylated cholesterol into its membrane with the cholesterol-α-glucosyl-transferase (Cgt) (see 1.1.6).[72] It has further been stated that cholesterol can augment the binding of ANXA2, ANXA5 and ANXA6 to phospholipids.[85] Therefore the role of cholesterol in the ANXA5 binding of *H. pylori* was investigated.

3.4.1 The growth medium of *Helicobacter pylori* has no effect on the Annexin A5 binding

Normally in this study *H. pylori* was grown on cholesterol agar plates when used for experiments (see 2.2.1.1). To test whether the cholesterol affects the binding of ANXA5, the Annexin binding assay was additionally performed with bacteria that had been cultivated on either serum or blood agar plates (as *H. pylori* is quite commonly grown on Colombia blood agar plates). Although those media contain cholesterol, unbound cholesterol is presumably not as consistently present as in cholesterol agar plates.[142]

The histograms (see Figure 22A) suggest that bacteria growing on cholesterol agar plates bound the most ANXA5. However, the quantitative analysis of the data showed no significant difference between bacteria grown on different plates (see Figure 22B).

It was therefore necessary to perform additional experiments to further investigate the role of cholesterol in the ANXA5 binding of *H. pylori* (see 3.4.2).

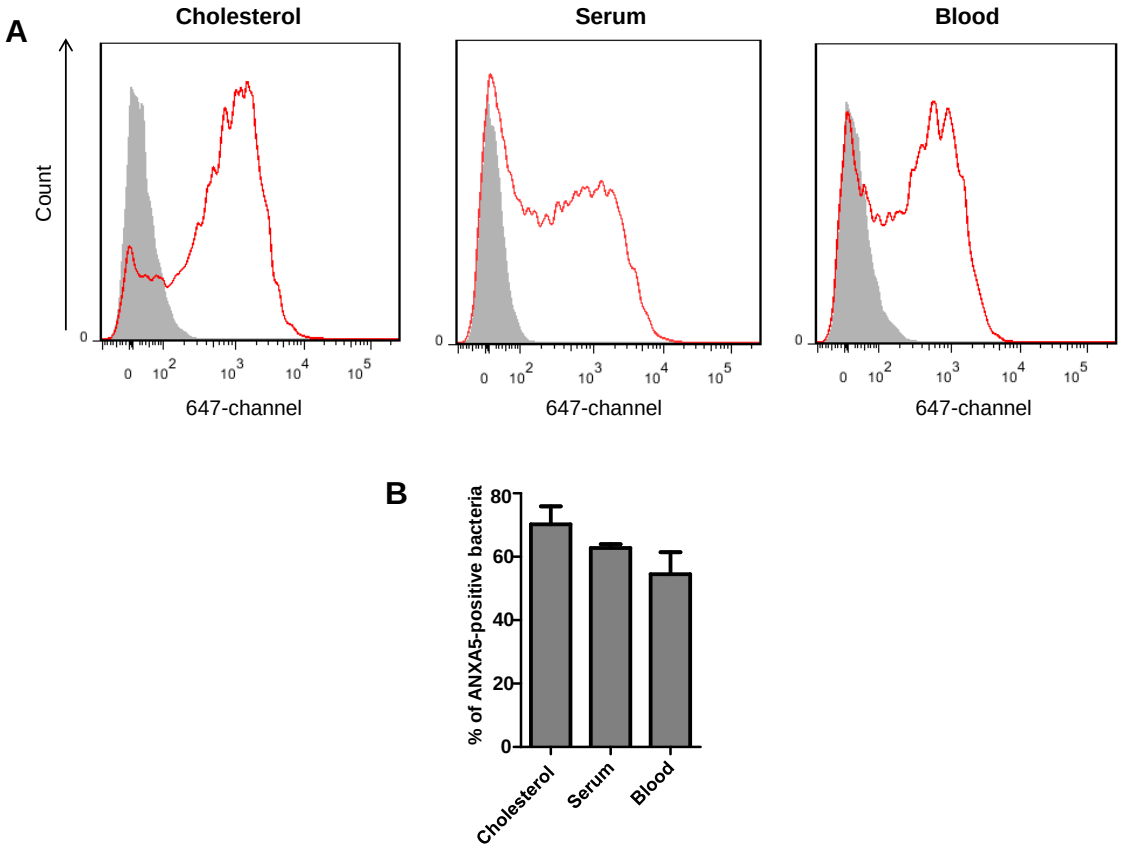


Figure 22 Effect of growth medium on *Helicobacter pylori* binding Annexin A5 (Cholesterol vs. Serum vs. Blood agar)

The Annexin binding assay was performed using *H. pylori* P12 GFP strain, which had been cultivated either on cholesterol, serum or blood agar plates (see 2.2.1.1) and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.

(A) Representative histograms of the 647-fluorescence of the bacteria grown on cholesterol, serum or blood agar plates (left to right). *H. pylori* only is shown in grey and with ANXA5 addition in red.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group is displayed here +/- SEM. Statistical analysis was done with one-way ANOVA (p=0.1832); n=3.
* p<0.05; ** p<0.01; *** p<0.001

3.4.2 Cholesterol-α-glucosyltransferase is not necessary for the Annexin A5 binding

To further evaluate the role of cholesterol for the ANXA5 binding of *H. pylori*, Cholesterol-α-glucosyltransferase (Cgt) mutants were tested. Cgt mutants are not able to incorporate cholesterol in the bacterial membrane. If the exploited cholesterol would play an important role in the *H. pylori*–ANXA5 interaction, the ANXA5 binding should be decreased for the Cgt mutant.

The Annexin binding assay was performed and the P12 wildtype was compared to a P12Δ*cgt* strain. Both the histograms and the quantitative analysis show that there was no significant difference in the ANXA5 binding of the bacteria (see Figure 23).

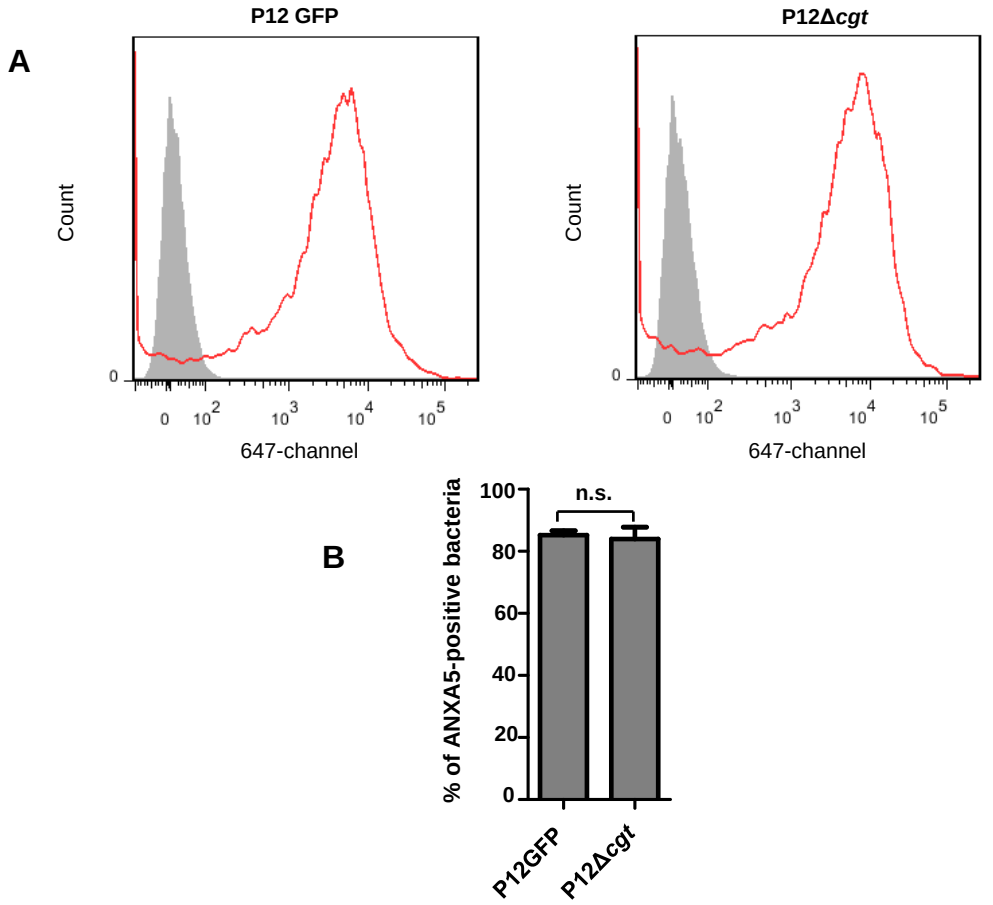


Figure 23 Role of cholesterol-α-glucosyltransferase (Cgt) for the binding of *Helicobacter pylori* to Annexin A5

The Annexin binding assay was performed using either *H. pylori* P12 GFP or a *cgt* mutant of P12 and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.

(A) Representative histograms of the 647-fluorescence of the *H. pylori* wildtype (left) and the *cgt* mutant (right) without (grey) and with (red) ANXA5-A647 addition.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are shown here with SEM. Unpaired t-test was performed, p=0.7581; n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

It can therefore be concluded from both experiments that the cholesterol metabolism does most likely not play an important role in the interaction of *H. pylori* and ANXA5.

3.5 Substantial effect of different growth conditions of *Helicobacter pylori* on the Annexin A5 binding – Solid vs. liquid culture

To evaluate the effect of different growth conditions, the ANXA5 binding of *H. pylori* that was either grown in liquid culture or on agar plate was compared. For this study *H. pylori* was mostly grown on agar plates, but it can also be cultivated in liquid culture. This might have an effect on the lipid distribution or on the "fitness" of the bacteria, and as a consequence could change the ANXA5 binding.

Therefore, an overnight culture of bacteria in Brucella broth and cholesterol was prepared and the Annexin binding assay (see 2.2.1.7) was performed.

Figure 24 illustrates representative histograms of the ANXA5-fluorescence of *H. pylori* that had been grown in either solid (A) or liquid (B) culture. The curve of the bacteria that grew in liquid culture is considerably shifted to the left as compared to the bacteria grown on plate, indicating that the ANXA5-binding capacity is clearly reduced. The quantitative analysis supports this initial impression. While nearly ~50 % of bacteria that grew on plate bound ANXA5, only ~7 % of bacteria grown in liquid culture did.

To exclude any interfering effect from the liquid culture medium on the binding, a control was performed. It could be observed that the ANXA5 binding of bacteria, which had been cultivated on agar plates, did not change, when liquid culture medium was added to the assay (see Figure 24C). These data suggest that it is highly unlikely that the liquid culture medium per se had any effect.

Taken together these experiments show that *H. pylori* most likely changes its surface binding characteristics depending on whether it was grown in liquid culture or on agar plates. These changes apparently greatly influence the binding of ANXA5 (see 4.1).

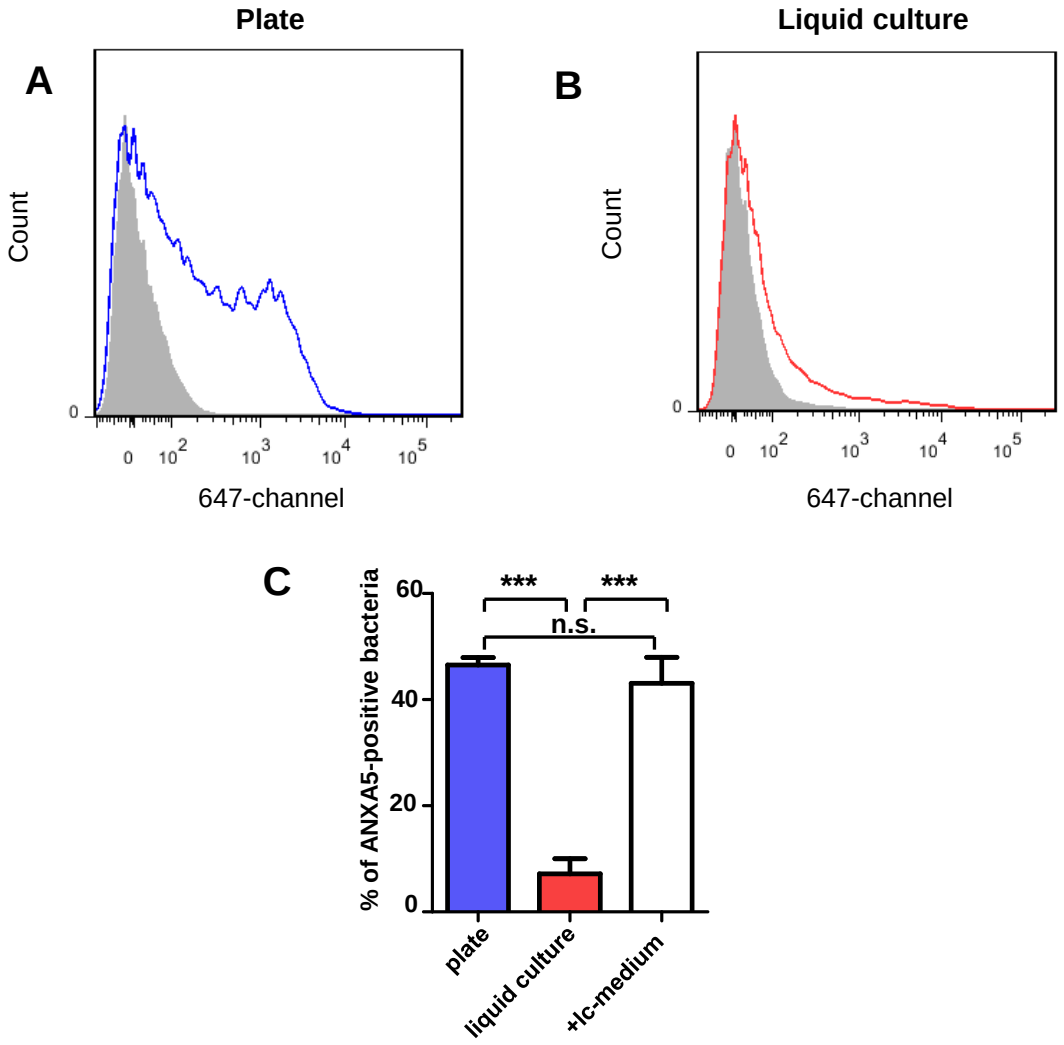


Figure 24 Effect of growth medium for *Helicobacter pylori* binding Annexin A5 (solid vs. liquid culture)

The Annexin binding assay was performed using *H. pylori* P12 GFP strain, which had been cultivated either on cholesterol agar plates or in liquid culture, and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.
(A-B) Representative histograms of the 647-fluorescence of the bacteria grown on plate (A) or in liquid culture (B). *H. pylori* only is shown in grey and with ANXA5 addition in blue (A) and red (B), respectively.
(C) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are displayed here +/- SEM. Bacteria grown on plate (blue) or in liquid culture (red) are shown as well as bacteria grown on plate with addition of liquid culture medium (lc-medium; white) to the assay as control. Statistical analysis was done with one-way ANOVA (p=0.0003) and Tukey's multiple comparison post test; n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

3.6 Role of *Helicobacter pylori´s* pathogenicity factors on binding Annexin A5

Two of the most important pathogenicity factors of *H. pylori* are the Cytotoxin-associated gene A (CagA) and the Vacuolating cytotoxin A (VacA) (see 1.1.3). Both have been associated with phosphatidylserine (PS), a common ligand of annexins.[29,151,152] It was therefore relevant to investigate whether they influence the interaction of *H. pylori* with ANXA5. Single mutants of CagA and VacA already existed in the P12 GFP strain, but it was also interesting to find out if a double mutant might have an effect on the binding of ANXA5. Therefore, a transformation was performed to create a double mutant.

3.6.1 Successful creation of a P12Δ*vacA*Δ*cagA* double mutant

H. pylori is naturally competent for DNA transformation[143] and this ability was used for the deletion of the *cagA* gene. The P12Δ*vacA*Δ*cagA* mutant was created by inserting a kanamycin cassette with flanking regions of *cagA* (see 2.1.6) in the P12Δ*vacA* strain expressing GFP.

The transformation was confirmed both on DNA and on protein level.

Figure 25A shows the results from PCR. With the primers used (see 2.1.7) the CagA product from the control strains with the normal CagA (P12wt and P12Δ*vacA*) should be around 5400 bp, which was confirmed with the agarose gel (see arrowhead in Figure 25A). The recombination event with the plasmid exchanges the normal *cagA* for a Kanamycin cassette (~3,000 bp) with flanking regions of c*agA*. The resulting product is much smaller (~4,000 bp), which can be seen for both the already existing P12Δ*cagA* strain as well as for the created double mutants (see arrow in Figure 25A).

The complementary Western blot analysis confirmed the mutation on protein level (see Figure 25B). While there was a signal from the α-CagA antibody for the P12 and P12Δ*vacA* strain, no signal could be detected for either P12Δ*cagA*, the Tx30a strain (which is naturally lacking *cagA*) or the created double mutant.

These experiments confirmed the successful creation of a P12Δv*acA*Δc*agA* double mutant. The presence of GFP protein was verified by microscopy (data not shown).

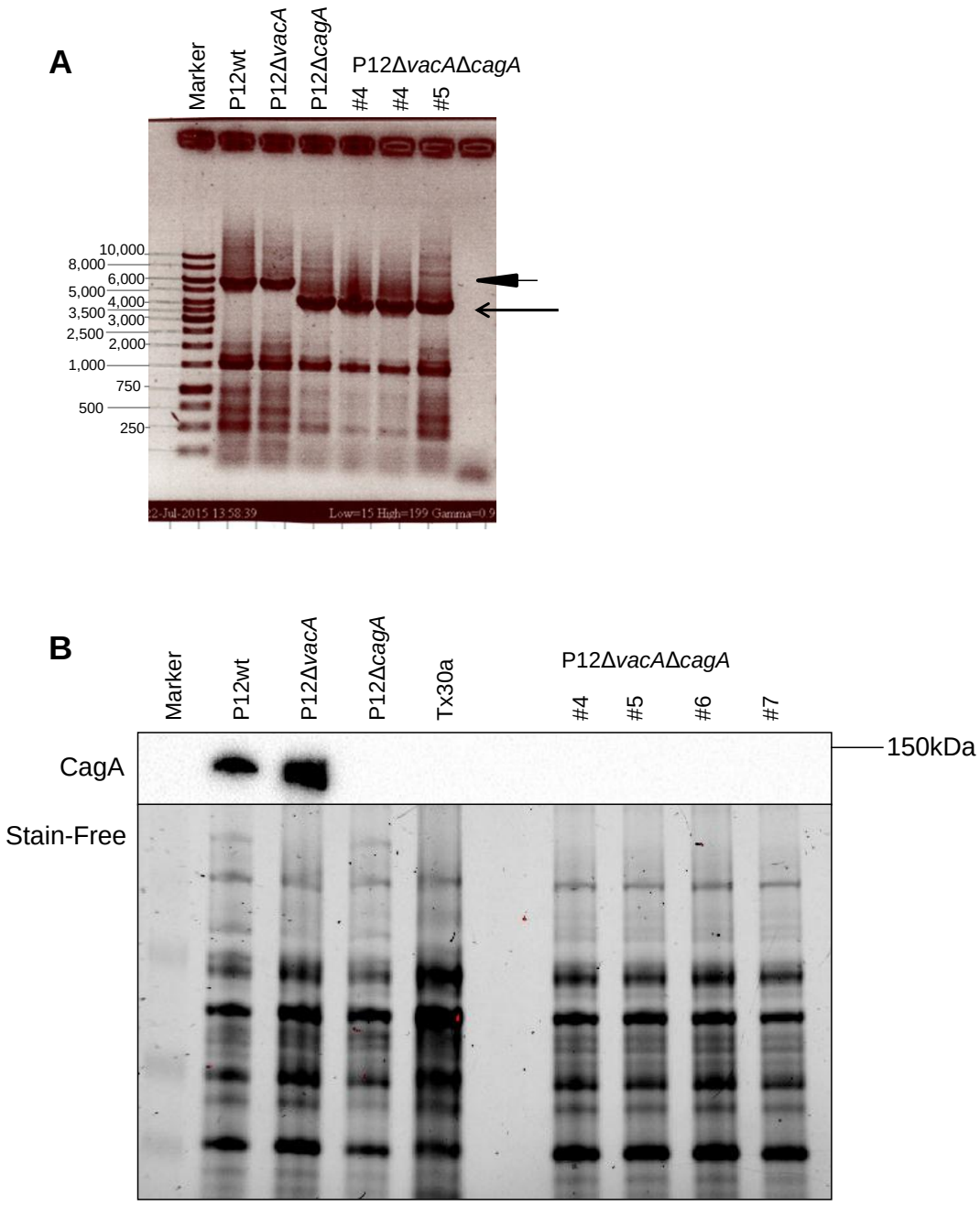


Figure 25 Confirmation of the P12Δ*cagA*Δ*vacA* GFP double mutant

(A) On DNA level a PCR for *cagA* was performed and the PCR products were loaded to an agarose gel. The marker in the left lane indicates base pairs. Displayed here are the P12 wildtype strain, single mutants with Δc*agA*GFP or Δv*acA*GFP and the created double mutant Δ*cagA*Δ*vacA*GFP (clone #4 and #5; #4 was loaded twice). Arrow head and arrow indicate the different sizes of products depending if the strain has the normal *cagA*-gene (arrow head) or not (arrow). All bands below 2,000 bp are considered as unspecific background signal.
(B) On protein level a Western blot was done with the α-CagA antibody AK299 to check whether the mutants express the CagA protein. The double mutants (clone #4-7) were compared to P12wt, Δ*vacA*GFP or Δ*cagA*GFP single mutants and Tx30a (c*agA*-negative *H. pylori* strain). The Stain-Free is shown as a loading control.

3.6.2 Annexin A5 binding is reduced in *Helicobacter pylori vacA* mutants, but unchanged in *cagA* mutants

After successful generation of the P12Δ*vacA*Δ*cagA*GFP double mutant the Annexin binding assay (see 2.2.1.7) was performed and the *H. pylori* strain P12 GFP was compared to the P12 Δ*cagA*-, Δ*vacA*- and Δ*vacA*Δ*cagA*-mutants. The analysis was performed by flow cytometry and the FlowJo software (see 2.2.6.1).

Figure 26A shows representative histograms. Together with the quantitative analysis they show that less percent of the bacteria from the Δ*vacA*- and the Δ*vacA*Δ*cagA* double-mutant bind ANXA5 as compared to the wildtype, while there is no significant difference between the Δ*cagA*-mutant and the wildtype (Figure 26B).

The findings suggest that it is not important for the capability of *H. pylori* to bind ANXA5 whether it expresses CagA or not. In contrast, VacA might play an important role in the *H. pylori*-ANXA5 interaction. When VacA is missing fewer bacteria bind ANXA5.

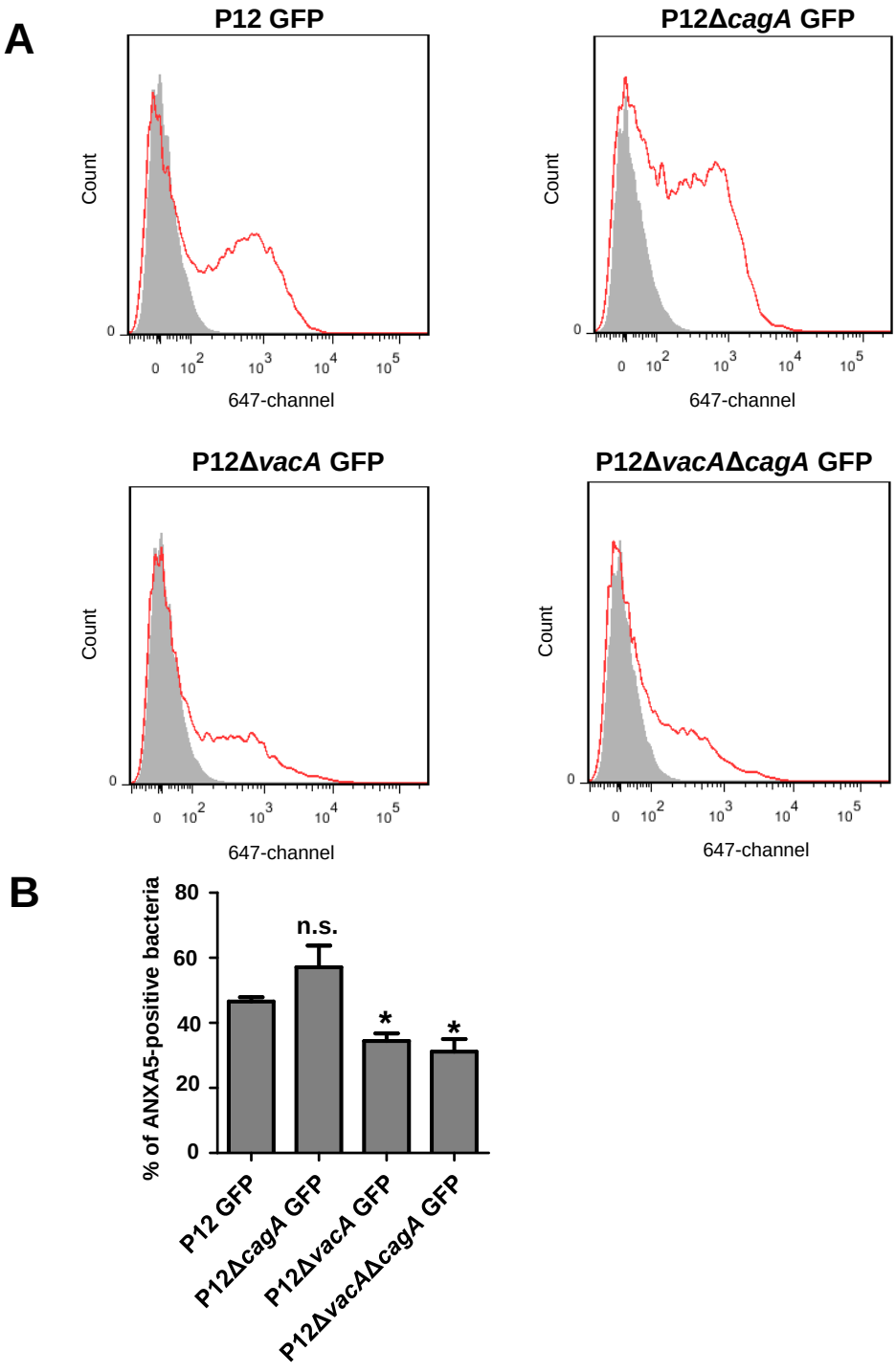


Figure 26 Different *Helicobacter pylori* mutants binding Annexin A5

The Annexin binding assay was performed using different *H. pylori* mutants and Alexa Fluor® 647-tagged ANXA5. Analysis was performed using flow cytometry.

(A) Representative histograms of the 647-fluorescence of the *H. pylori* wildtype and different mutants without (grey) and with (red) ANXA5-A647.
(B) With FlowJo the flow cytometry data were gated to the ANXA5-positive events; the mean percentage of bacteria in this group are shown here with SEM. Unpaired t-test was performed against P12 GFP, because for testing the hypothesis it was only interesting how one mutant behaved as compared to the wildtype and not compared to the other mutants; p=0.1976 for Δ*cagA* mutant, p=0.0107 for Δ*vacA* mutant and p=0.0202 for Δ*vacA*Δ*cagA* double mutant; n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

3.7 Potential functions of the interaction of *Helicobacter pylori* with Annexin A5

3.7.1 Annexin A5 does not affect the binding of *Helicobacter pylori* to AGS cells

H. pylori can cause various effects in its host cells. An important step for the bacterium is to bind to the host cell membrane to deliver one of its pathogenicity factors, CagA, into the cell via the type IV secretion system (T4SS). Because ANXA5 is known to interact with various membrane lipids, it could interfere with the bacteria-cell interaction and cause a change in the binding. It was therefore relevant to test whether ANXA5 has any effect on *H. pylori´s* capability to bind to the host cells.

To evaluate this question AGS cells were infected with a fluorescent *H. pylori* strain. In the first assay ANXA5 was added simultaneously with the bacteria to the AGS cells. In a second assay *H. pylori* was incubated for one hour with ANXA5 (or incubated alone as a control) before the AGS cells were infected. After the infection and one hour of incubation the cells were washed and harvested. Analysis was then performed in the flow cytometer. The fluorescence signal was measured and used as an indicator that bacteria had bound to the cells.

The binding of the bacteria to the AGS cells did not demonstrate any significant difference whether ANXA5 had been added or not. Whether ANXA5 was added simultaneously (Figure 27A) or whether the bacteria were pre-incubated with it (Figure 27B) did not make any difference either.

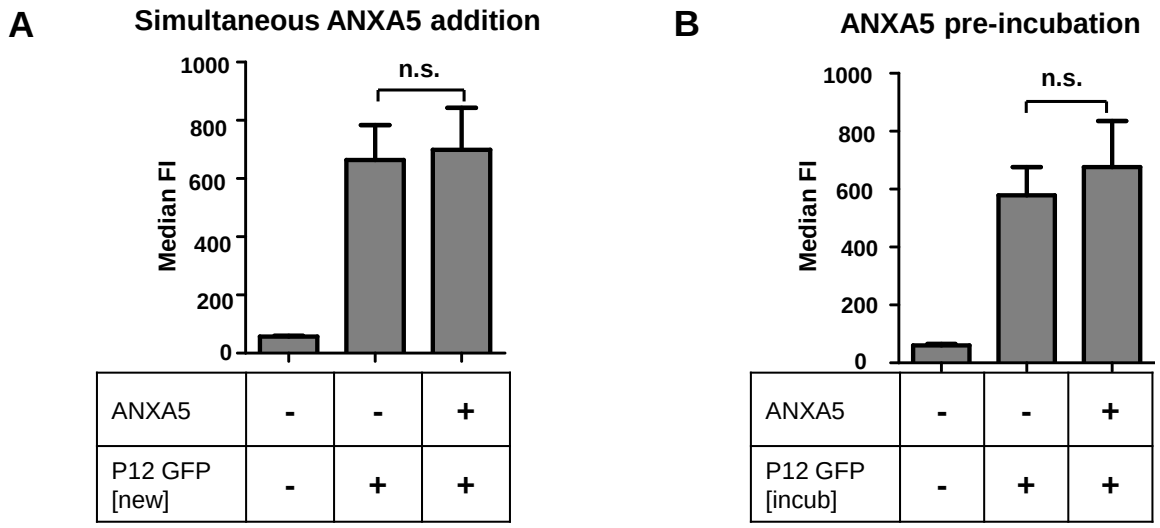


Figure 27 Effect of ANXA5 addition for the binding of *Helicobacter pylori* on AGS cells

AGS cells were infected with P12 GFP. The bacteria had either been pre-incubated with ANXA5 (B) or ANXA5 was added simultaneously with the bacteria to the cells (A). Analysis was performed using flow cytometry.
The 488-fluorescence of the AGS cells was measured after the infection and several washing steps. As the bacteria were expressing GFP this 488-fluorescence represents the bacteria that bound to the AGS cells. The mean value with SEM of the median fluorescence intensity of the 488-channel is displayed in the graphs.
Statistical analysis was done with one-way ANOVA (p=0.0037 (A) and p=0.0142 (B)) and Tukey's multiple comparison post test; n=3-4.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

3.7.2 Annexin A5 reduces the CagA translocation of *Helicobacter pylori* in AGS cells

H. pylori can inject its pathogenicity factor CagA into host cells via the T4SS. To do so, the bacterium has to dock to the host cell´s membrane. Once the CagA is in the host cell, it is phosphorylated on a tyrosine residue and has various effects on the cell (see 1.1.3).

Hatakeyama et al. reported that the addition of ANXA5 to an infection of *H. pylori* with AGS cells resulted in a ~20 % decrease of CagA translocation.[29] The following experiments were performed to see whether these results could be verified and to see if ANXA5 really influences the CagA translocation of *H. pylori* into AGS cells.

AGS cells were infected with *H. pylori*. In the first assay ANXA5 was added simultaneously with the bacteria to the AGS cells. In a second assay *H. pylori* was incubated for one hour with ANXA5 (or incubated alone as a control) before the AGS cells were infected. The analysis was performed by Western blotting and the phosphorylated CagA could then be detected (see 2.2.2.3.4).

When ANXA5 was added simultaneously with the bacteria to the AGS cells, a trend could be seen that the CagA translocation was reduced by ~22 %, though the difference was not

statistically significant (see Figure 28A). However, when *H. pylori* was pre-incubated with ANXA5 and was then used to infect the AGS cells, the CagA translocation was significantly reduced (by ~33 % on average) (see Figure 28B).

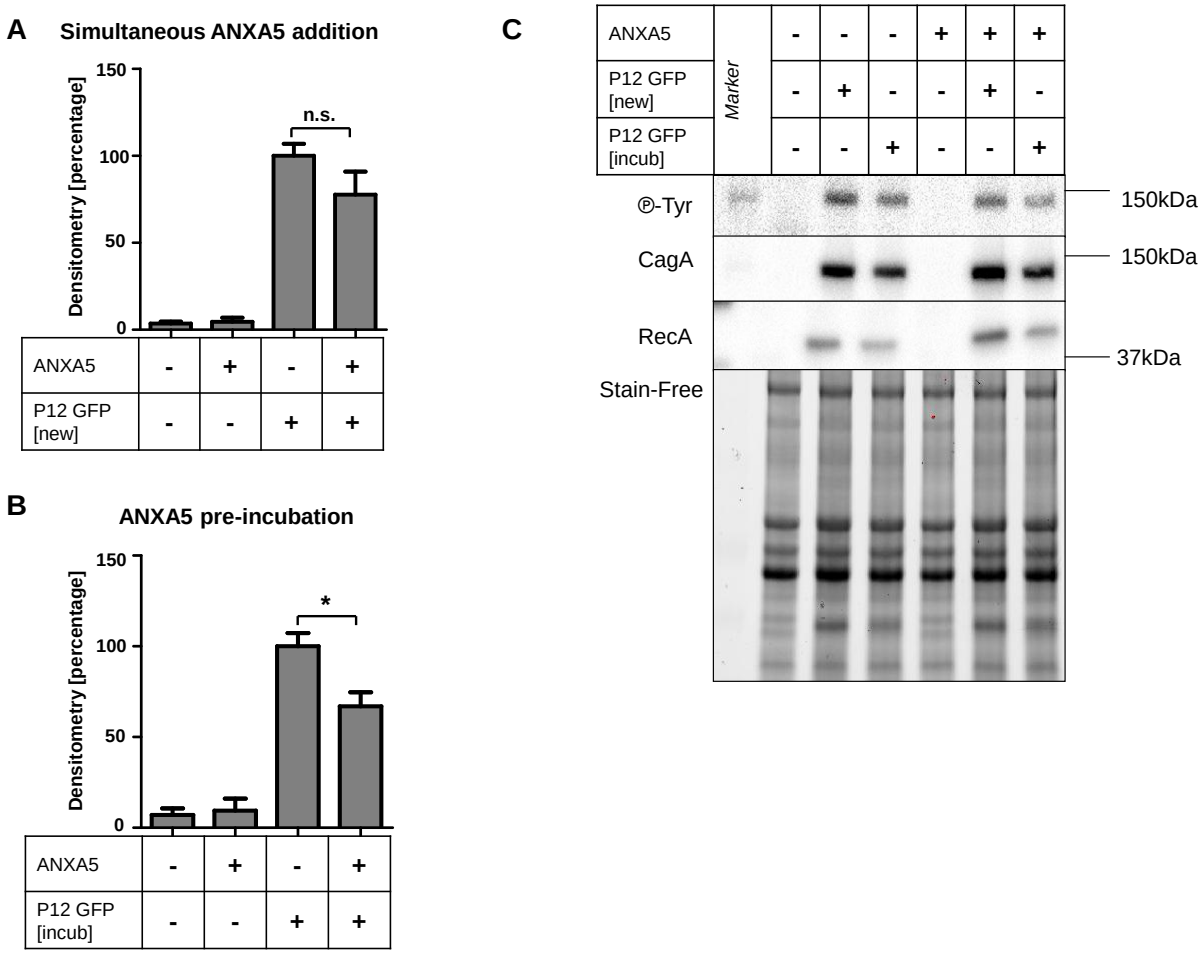


Figure 28 Effect of Annexin A5 on the CagA translocation of *Helicobacter pylori*

AGS cells were infected with the *H. pylori* strain P12 GFP. Optionally ANXA5 was added to the infection either simultaneously (A) or bacteria were used, that had been pre-incubated with ANXA5 (B). After infection and incubation the cells were harvested and lysates were analysed with Western blot. *H. pylori* can inject it´s pathogenicity factor CagA into the AGS cells, where it is phosphorylated. This phosphorylated CagA can be detected with an anti-phosphotyrosin antibody. Semi-quantitative analysis was done (see 2.2.6.2).
A reduction can be seen, when ANXA5 was added. Statistical analysis was performed with one-way ANOVA ($p<0.0001$ for (A) and (B)) and Tukey's multiple comparison post test; n=4.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$, n.s. = no significant difference
(C) Representative blots of phosphorylated CagA (Ⓟ-Tyr), CagA and RecA as well as the Stain-Free image are shown.

These data not only confirm the observations by Hatakeyama et al.,[29] but also show that pre-incubation of bacteria with ANXA5 enhances this effect.

3.7.3 No effect of Annexin A5 on the IL-8 induction in AGS cells by *Helicobacter pylori*

CagA is not the only mediator that causes effects in host cells after an infection with *H. pylori*. Moreover, there is an induction of IL-8 production caused by the T4SS and CagA (see 1.1.3). As there was an effect of ANXA5 on the CagA translocation (see 3.7.2) and there is a connection between the T4SS, CagA and IL-8, it was interesting to test whether ANXA5 also had an effect on the IL-8 induction in AGS cells by *H. pylori*.

To address this question AGS cells were infected with *H. pylori*. In the first assay ANXA5 was added simultaneously with the bacteria to the AGS cells. In a second assay *H. pylori* was incubated for one hour with ANXA5 (or incubated alone as a control) before the AGS cells were infected. After the infection and three hours of incubation, the supernatant of the cells was harvested and used for ELISA to detect the IL-8 (see 2.2.2.3.5).

No difference in the IL-8 induction could be observed whether ANXA5 was added together with *H. pylori* or not. It did not matter whether the ANXA5 was added simultaneously or whether the bacteria were pre-incubated with it (see Figure 29A and B, respectively). Uninfected cells also showed a certain background signal, though it did not make any difference whether nothing was added or only ANXA5.

It could therefore be shown that the IL-8 induction of AGS cells upon infection with *H. pylori* does not change whether ANXA5 is added or not.

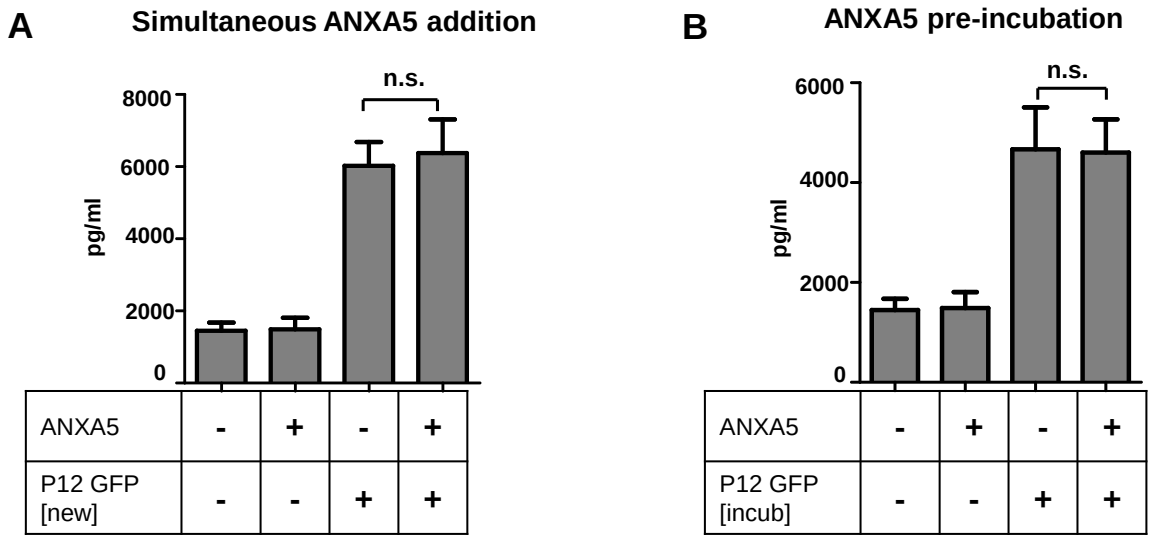


Figure 29 Effect of Annexin A5 on the IL-8 induction of *Helicobacter pylori*

AGS cells were infected with the *H. pylori* strain P12 GFP. Optionally ANXA5 was added to the infection either simultaneously (A) or bacteria were used, which had been pre-incubated with ANXA5 (B). After infection and incubation supernatants from the cells were harvested. *H. pylori* can induce IL-8 production in AGS cells and this was measured with ELISA of these supernatants (see 2.2.2.3.5).
Statistical analysis was performed with one-way ANOVA (p=0.0008 for (A) and p=0.0040 for (B)) and Tukey's multiple comparison post test; n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference

3.8 *Helicobacter pylori´s* binding of other annexins

The focus of this study was laid on ANXA5 for various reasons: The interaction of ANXA5 with other gram-negative bacteria and further microorganisms has been described before (see 1.2.4). Furthermore, ANXA5 is widely available in various forms (for example with fluorophore-tags). Different members of the annexin family have some properties and functions in common, but also differ in other aspects (see 1.2.2). It was therefore interesting to expand this study and test the interaction with other annexins: First, to further evaluate if the interaction of *H. pylori* and ANXA5 is specific for ANXA5 or if it can also be seen with other annexins. Secondly, the results could be used to further illuminate possible functions of the *H. pylori*-ANX interaction and clarify which part of ANX is responsible for the interaction.

ANXA1 and ANXA2 were selected for the test because of different considerations: In contrast to ANXA5, their N terminus, which is the variable part of an ANX protein, is much larger. They were described to play a role in the carcinogenesis of gastric cancer and ANXA2 was found to be up-regulated in *H. pylori*-associated gastric cancer (see 1.2.3). Moreover, ANXA1 and ANXA2 can interact with bacterial lipid A, and ANXA2 was shown to be essential for the infection of a variety of different human viruses (see 1.2.4).

To analyse the *H. pylori*-ANXA1 and -ANXA2 interaction the Annexin binding assay (see 2.2.1.7.1) was performed with ANXA1 and different *H. pylori* wildtype strains, as well as with ANXA2 and the P12 wildtype strain. The samples were prepared and loaded to a single gel system polyacrylamide gel (see 2.2.4.1). ANXA1/A2 were also prepared without *H. pylori* and loaded as a positive control. The protein was detected by immunodetection with anti-ANXA1- and anti-ANXA2-antibodies, respectively.

Figure 30A shows a representative blot for the ANXA1 binding of different *H. pylori* wildtype strains. All three tested strains (P12, 26695 and Tx30a) bound ANXA1, similar to the binding of ANXA5 (compare to Figure 11C). Moreover, the P12 strain could be shown to interact with ANXA2 (see Figure 30B). The binding of other *H. pylori* strains to ANXA2 still needs to be further evaluated.

It could therefore be shown that the *H. pylori*-ANX interaction is not restricted to ANXA5, but also occurs with other annexins, namely ANXA1 and ANXA2.

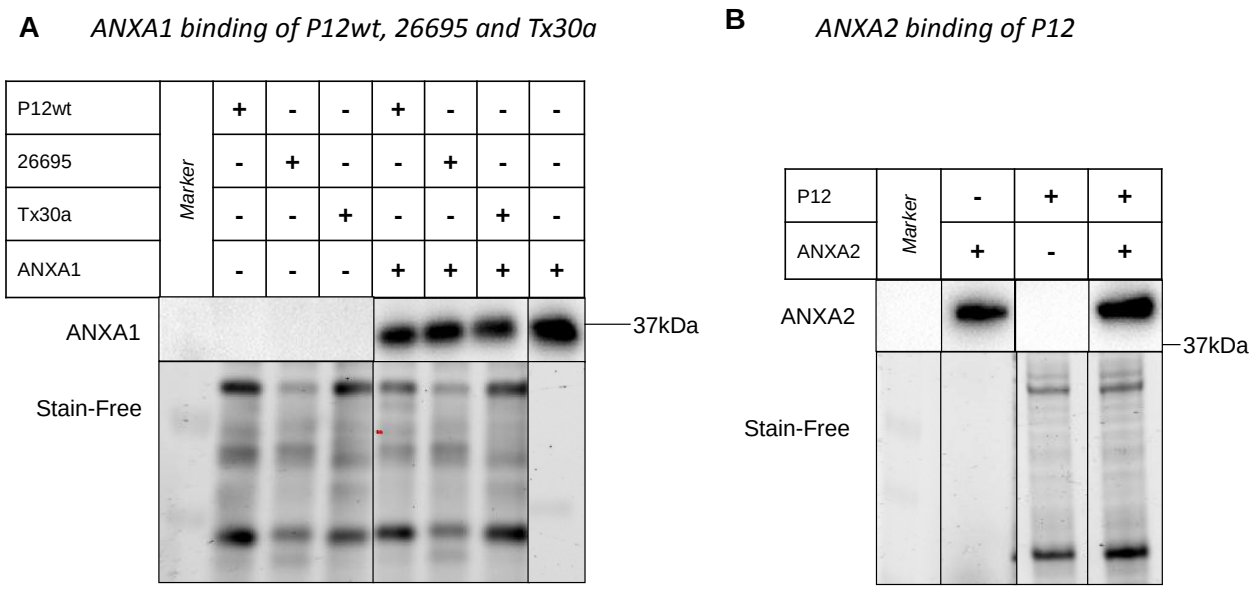


Figure 30 *Helicobacter pylori* binding Annexin A1 and Annexin A2

The Annexin binding assay was performed using different *H. pylori* strains and ANXA1 (A) or ANXA2 (B). Analysis was performed using Western blot.

(A) *H. pylori* wildtype strains P12, 26695 and Tx30a were incubated with ANXA1, the samples were prepared and then gel electrophoresis and Western blot were performed. With an anti-ANXA1 antibody the ANXA1 was detected. ANXA1 only was used as a positive control. The Stain-Free is shown as a control for the amount of protein loaded. n=3; a representative example is shown here. Experiments were performed by LJ.
(B) *H. pylori* P12 wildtype strain was incubated with ANXA2, the samples were prepared and then gel electrophoresis and Western blot were performed. With an anti-ANXA2 antibody the ANXA2 was detected. ANXA2 only was used as a positive control. The Stain-Free is shown as a control for the amount of protein loaded. n=2; a representative example is shown here. Experiments were performed by LJ.

3.9 Physiological relevance of annexins in *Helicobacter pylori* infection

Because a novel interaction was examined in this study many experiments were performed in a strict laboratory setting with “artificially” added annexins. To begin to understand the physiological relevance of this interaction, it was important to go one step further and have a look at the more complex infection processes as well as analyse the naturally present annexins of the gastric cells.

It was thus interesting to confirm the presence of annexin in gastric cells and tissue and to find out how the expression level of annexins changed upon infection with *H. pylori* both in cell culture and in human gastric tissue.

3.9.1 Annexin A2 expression in AGS cells does not change upon infection with *Helicobacter pylori*

The standard cell line used in our laboratory as a cell culture infection model is the human gastric adenocarcinoma (AGS) cell line. Preliminary data showed that AGS cells contain ANXA2, but not ANXA1 or ANXA5 (data not shown; LJ). Therefore, it was examined whether ANXA2 was up- or down-regulated upon infection with *H. pylori*.

AGS cells were infected with the *H. pylori* strain P12 GFP or a mutant lacking the *cag*-PAI (P12*ΔPAI*GFP) (see 2.2.2.3.6). Subsequently, ANXA2 expression in AGS cells, possible changes upon infection with *H. pylori* and the dependency on the *cag*-PAI of *H. pylori* were analysed. ANXA2 could be detected in the AGS cell lysates. There was no significant difference in the ANXA2 expression, whether AGS cells had been infected with *H. pylori* or not (see Figure 31). Moreover, it did not make any difference whether a pathogenic strain was used or whether the bacteria were lacking the PAI.

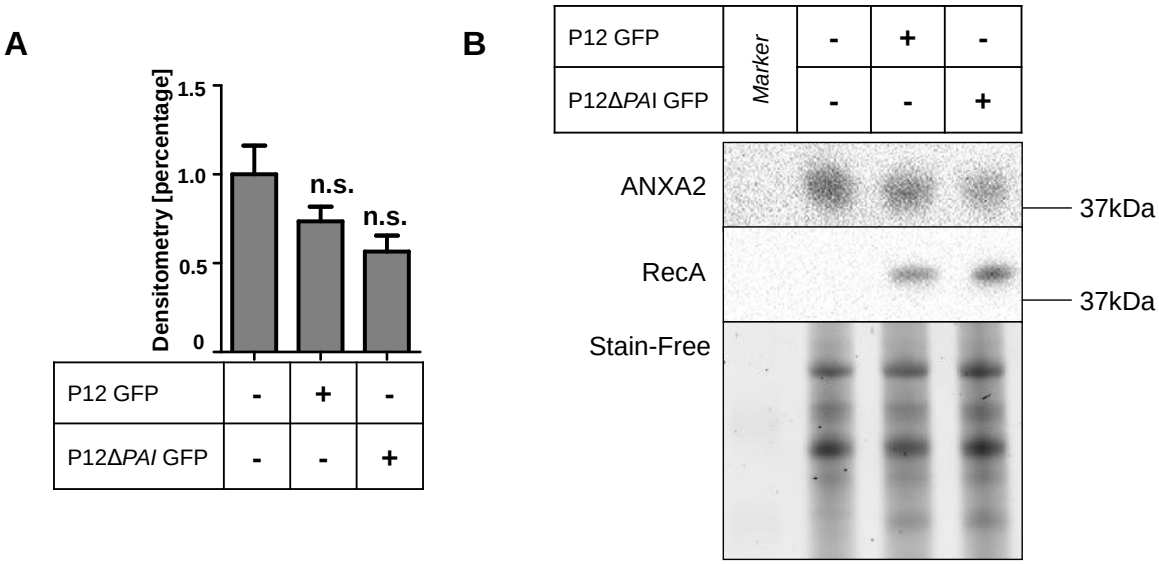


Figure 31 Annexin A2 expression in AGS cells upon infection with *Helicobacter pylori*

AGS cells were infected with the *H. pylori* strains P12 GFP or P12Δ*PAI* GFP. After incubation for 3 hours cells were put on ice and washed twice to remove unbound bacteria. AGS cells were then harvested and lysates were analysed with Western blot. ANXA2 expression in AGS cells and changes upon infection with *H. pylori*, as well as the dependency of potential changes on the *cag*-PAI of *H. pylori*, were analysed.
(A) Semi-quantitative analysis was performed (see 2.2.6.2). AGS expression of ANXA2 could be shown, but no significant difference upon *H. pylori* infection or between the strains.
Statistical analysis was performed with one-way ANOVA (p=0.099) and Dunnett's multiple comparison test; n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference
(B) Representative blots of ANXA2 and RecA (bacterial protein that was used to confirm the presence of bacteria) as well as the Stain-Free (control for the amount of protein loaded) are shown.

3.9.2 Upregulation of Annexin A2 and A5 in human stomach biopsies upon H*elicobacter pylori* infection

Commercially available human gastric tissue biopsies (with and without *H. pylori* infection) were stained and analysed for ANXA2 and ANXA5 expression. For this purpose the tissue slides were deparaffinised, permeabilized and stained with DAPI and either anti-ANXA2- or anti-ANXA5-antibody (see 2.2.3). The analysis was then performed by confocal laser scanning microscopy and the quantification of the annexin expression in the tissue slides was done with Fiji ImageJ software (see 2.2.6.3). The staining procedure was repeated for three tissue slides each and images were obtained of five different, representative areas per slide.

Though a trend can be seen that the ANXA2 expression is higher in *H. pylori*-infected gastric tissue (~38 % compared to ~16 % in non-infected tissue), the difference is not significant (see Figure 32A). In contrast, the ANXA5 expression is significantly enhanced from ~2.6 % to ~6.6 % in gastric tissue after *H. pylori* infection (see Figure 32B). It is noticeable that a higher percentage of cells in the stomach biopsies express ANXA2 as compared to ANXA5. This can also be observed in the representative sample images in Figure 32C. Nuclei are shown in blue (DAPI staining) and ANXA2/A5 are shown in green. It was not stated on the slides from which region of the stomach the samples were taken and the obtained micrographs give the impression that uninfected samples come from a different region than the ones with an *H. pylori* infection. See 4.3 for further discussion.

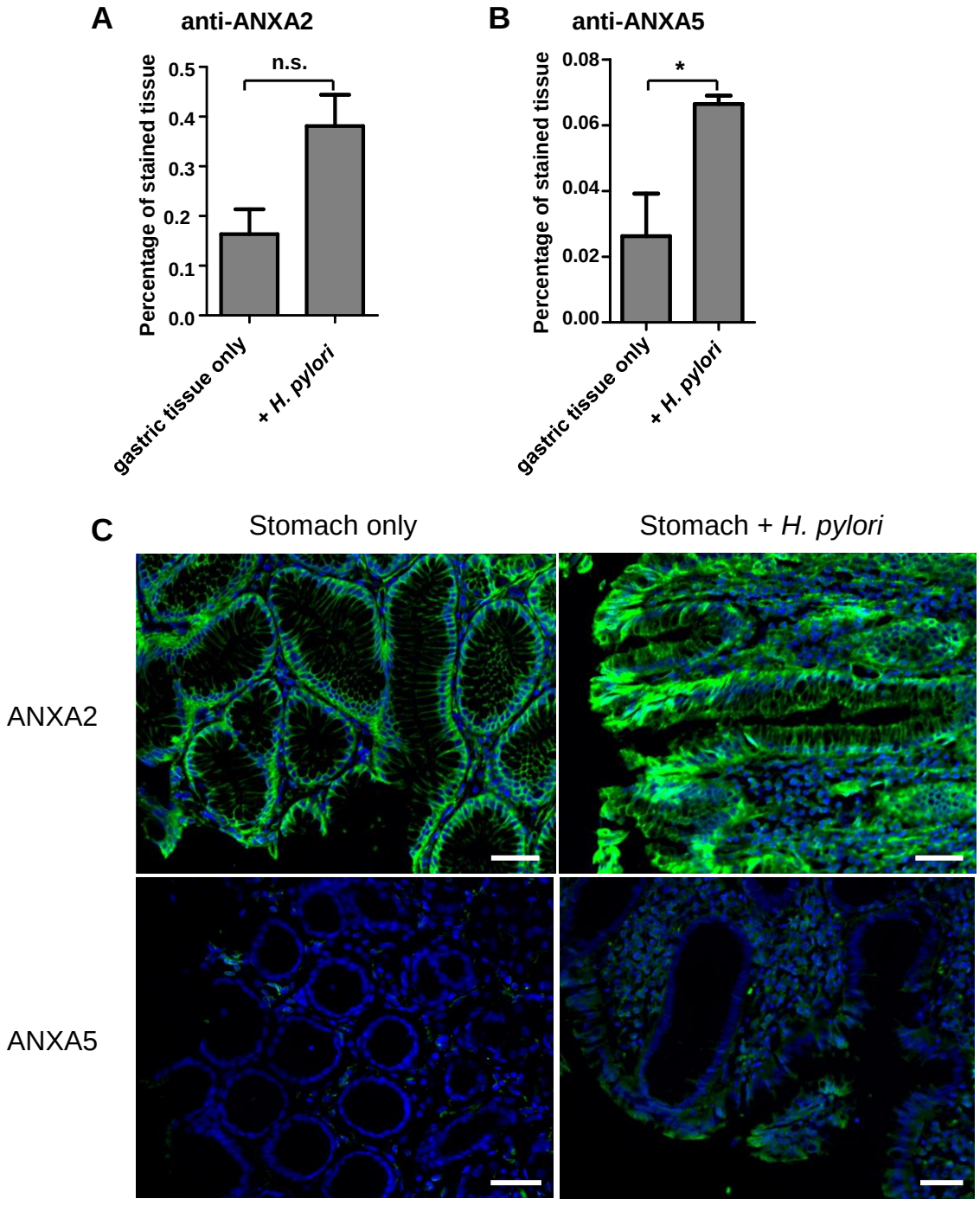


Figure 32 Expression of Annexin A2 and A5 in human gastric tissue samples with and without *Helicobacter pylori* infection

Tissue samples from the human stomach with and without *H. pylori* infection were stained with DAPI (blue) and either anti-ANXA2 or ANXA5 antibodies (green). The percentage of stained tissue was used as indication for the expression of the respective annexins (for analysis details see 2.2.6.3). Upon *H. pylori* infection the expression of ANXA2 (A) and ANXA5 (B) was enhanced. This increase was significant for ANXA5.
Unpaired t-tests were performed and showed a significant difference for ANXA5 ((A): p=0.0381) and a nearly significant difference for ANXA2 ((B): p=0.0538); n=3.
* p<0.05; ** p<0.01; *** p<0.001, n.s. = no significant difference
(C) Representative excerpts from images of the stained gastric tissue are shown, with and without *H. pylori* infection. Scale bar equals 50 µm. (DAPI=blue; ANXA2/A5=green)

4 Discussion

4.1 Lipid-mediated interaction of annexins and *Helicobacter pylori* and comparison to other bacteria

The main aim of this study was to identify a potential interaction between Annexins (ANXs) and *H. pylori* and, given that such an interaction exists, to further investigate the nature of this interaction.

This study shows for the first time that *H. pylori* interacts with ANXA5, which was confirmed by using various methods (see Figure 8 and Figure 9). This observation is consistent with previous data, which unintentionally showed a colocalisation of ANXA5 and *H. pylori* in confocal laser scanning microscopy (see Figure 34). As soon as the novel interaction between ANXA5 and *H. pylori* was identified and investigated in this study, an Annexin binding assay was standardized.

For the standardization, the cell culture media (RPMI and its serum complemented form, complete medium (CM)) were compared to a modified Annexin binding buffer (AnxBuf) (modified after Kenis et al.[138]). The ANXA5 binding was significantly reduced, when the assay was conducted in CM as compared to incubation in RPMI or AnxBuf (see Figure 12). CM consists of RPMI and 10 % foetal calf serum (FCS). FCS comprises a variety of contents that could potentially influence the ANXA5 binding of *H. pylori*. Furthermore, it is a biological product and could lead to huge variations between experiments when using different batches of FCS. This assumption was verified by performing the Annexin binding assay in RPMI complemented with different lots of FCS, showing a high variation in the ANXA5 binding of *H. pylori* between the different lots of FCS (data not shown). Moreover, it has been published that FCS can potentially be contaminated with viruses, and that proteins from the serum can possibly interact with bacterial membranes.[153,154] For the given reasons, it was decided to only use serum free solutions as incubation buffer for the Annexin binding assay. However, a comparison of the serum free solutions (AnxBuf and RPMI) showed no significant difference in the ANXA5 binding of *H. pylori* (see Figure 12). RPMI is a commercially available medium and did not have to be prepared in the lab, therefore allowing a better control of variations. Since both solutions were effective, and in order to reduce variability, RPMI was chosen as the standard incubation medium for the Annexin binding assay for this study. When an alternative was needed, experiments were performed as duplicates in both AnxBuf and RPMI.

Furthermore, the relevance of the incubation temperature was tested. The ANXA5 binding of *H. pylori* was slightly reduced when the incubation was performed at 4°C as compared to

37°C (see Figure 13). One possible explanation for this change is the reduction of *H. pylori´s* metabolism, reducing its movement or the capacity to react to the environment in order to interact with ANXs. Additionally, low temperatures can affect the membrane fluidity and its ability to create bindings.[150] For further experiments the incubation of bacteria with ANXs was performed at 37°C, as it corresponds better to the natural environment and physiology of *H. pylori* and its possible interactions in the human body.

Interactions between bacteria and ANXs can be affected by the concentration of both components. Therefore, the ideal ratio between *H. pylori* and ANXA5 was determined, first by testing different amounts of ANXA5. Concentration changes of ANXA5 show that the percentage of bacteria that bound ANXA5 remained constant, while the median fluorescence intensity (MFI) increased with a rising amount of ANXA5 (see Figure 15). Because the flow cytometry data in this study were mostly analysed by looking at the percentage of bacteria binding ANXA5 and as this was unchanged by the amount of ANXA5 used, it was decided to use lower amounts (2.5 or even 1.5 µl) of ANXA5 for future experiments. Secondly, no significant difference was detected when using different concentrations of bacteria, though there was a trend that the bacteria bound more ANXA5, when there were fewer bacteria (see Figure 14). This seems plausible as there is simply more ANXA5 that bacteria can bind. There were high variations between experiments, especially for the lower bacterial concentration (measured in OD_{550}, see 2.2.1.4), and it was therefore initially decided to use the highest concentration tested, OD_{550}=0.1 (~$3x10^7$ CFU), for further experiments. To confirm that this concentration was adequate for future experiments, the experiments with different amounts of bacteria were repeated by using a much higher quantity of ANXA5, whereby no difference could be observed in the binding (see Figure 16). The data suggest that a saturation effect takes place, which might make it more difficult to observe minor differences when other conditions are tested. In conclusion, it was decided to use lower amounts of ANXA5 and a reasonably high amount of bacteria, to ensure a consistent data outcome, a high sensitivity as well as economic efficiency.

ANXs most commonly either interact in a calcium-dependent way with lipids (mainly phospholipids) or calcium-independently with proteins (see Figure 33, I and II).[79,81] Both options have been described before for the interaction of ANXA5 with other microorganisms (e.g. [108,113,114]). It was therefore necessary to test whether the ANXA5 binding of *H. pylori* was lipid- or protein-mediated. It was hence first evaluated whether the binding is calcium-dependent, which would indicate a lipid binding. A next step was to see if the binding still takes place after denaturation of the bacterial proteins.

To test the calcium dependency of the ANXA5-*H. pylori* interaction, experiments were performed in different media by adding the chelator EGTA. EGTA is able to build a complex with and bind to divalent ions. It shows a high specificity for calcium ions, even in the presence of other divalent ions like magnesium.[155] The lack of binding in the absence of calcium (after adding EGTA) indicates that the *H. pylori*-ANXA5 binding is mainly calcium-dependent (see Figure 18). Although some bacteria (~8-16 %) still bound ANXA5 after EGTA addition, this could be a background binding caused by unspecific clustering. Another possible explanation could be that the amount of EGTA was not high enough or that EGTA was occupied by other ions and could hence not bind to, and consequently block, all calcium ions.

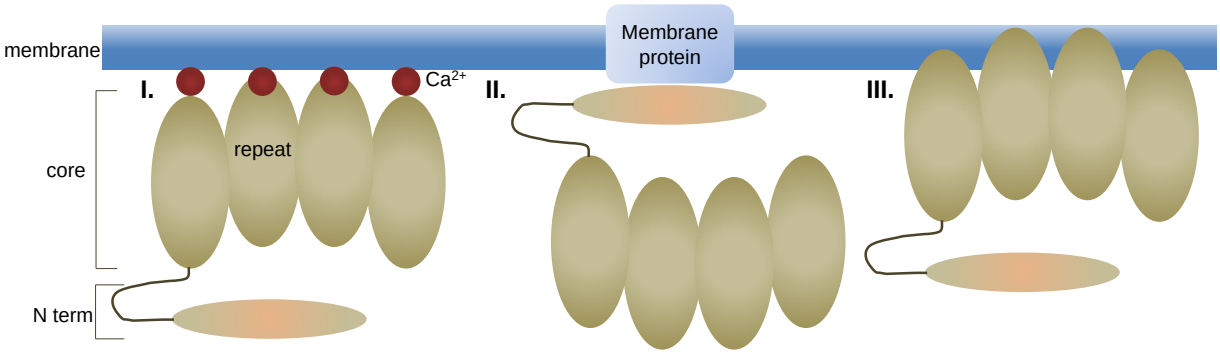


Figure 33 Membrane interactions of annexins

Annexins (ANXs) can interact with membranes in three different ways: I.) Through a calcium-dependent binding of their C-terminal domain to membrane lipids, II.) Through the N-terminal domain to membrane proteins and rarely, III.) Calcium-independently lipid-mediated (see 1.2.1). According to this study *H. pylori* and ANXA5 seem to interact as shown in I.).
Simplified scheme of the structure of membrane bound ANX proteins with its conserved protein core domain (core) with four homologous ANX repeat domains (repeat) and an N-terminal domain (N term). Calcium ions are shown in red (Ca^{2+}).
ANX structure adapted from Gerke, V. et al.[80]

To complementarily analyse the calcium dependency, it was tested how an increase of the calcium concentration affects the ANXA5 binding. As Figure 19 shows, more bacteria bind ANXA5 and the bacteria bind significantly more ANXA5, when more calcium ions are present. These findings, which show that the *H. pylori*-ANXA5 binding is calcium-dependent and enhanced upon the increase of the calcium concentration, support the hypothesis that the binding is lipid-mediated, as a protein-mediated interaction would not be calcium-dependent. Though ANXA5 can also bind to lipids in a calcium-independent way (Figure 33, III), this only occurs in acidic pH.[82] This possibility can therefore mostly likely be excluded, because the experiments were performed in neutral pH and the observed binding was calcium-dependent.[156] However, a calcium-independent, lipid-mediated interaction between *H. pylori* and ANXs cannot be ruled out in an acidic environment, and it is important to bear in mind that *H. pylori´s* natural habitat, the human stomach, is highly acidic.

To further verify that the binding occurs lipid- and not protein-mediated, it was tested whether the ANXA5 binding still took place when the proteins of *H. pylori* had been damaged. The protein denaturation was achieved by heat treatment and confirmed by evaluating the activity of the GFP protein of the bacteria (see Figure 20C). Denaturation of the proteins did not negatively affect the ANXA5 binding of *H. pylori* (see Figure 20A and B). The conclusion that can be drawn from these data is that the ANXA5 binding is independent of the intact conformation of *H. pylori* proteins. This supports the thesis that the binding is lipid-mediated, as it still occurs when proteins, as potential interacting partners, had been denatured.

The conserved C-terminal core domain of ANXs is responsible for the binding to phospholipids, while the N-terminus mediates the interaction with other proteins (see Figure 33).[79,81] It could be shown in this study that *H. pylori* does not only bind ANXA5, but also ANXA1 and ANXA2 (see Figure 30). ANXs differ greatly in their N-terminal domain. While ANXA5 has a short N-terminus, the ones from ANXA1 and ANXA2 are longer, consisting of up to 55 amino acids.[82] In contrast, the C-terminal domain between different ANXs is highly conserved. It is therefore probable that the binding occurs via the mutual C-terminus, as it is very similar for all tested ANXs. Other studies also came to the conclusion that, when different ANXs show the same behaviour, the C-terminus is more likely involved than the N-terminus.[85] This finding would complementarily strengthen the assumption that the *H. pylori*-ANXA5 interaction is lipid-mediated, as lipid interaction takes place via the C-terminus.

Although this study could show for the first time that *H. pylori* binds ANXA5, an interaction of ANXA5 and other bacteria has been described before. Rand et al. reported an interaction between ANXA5 and certain gram-negative bacteria (*Pseudomonas aeruginosa*, *Shewanella putrefaciens* and *Haemophilus influenzae*), but showed that the gram-positive bacteria that were tested (*Enterococcus faecalis*, *Streptococcus pyogenes* and *Streptococcus agalactiae*) did not bind ANXA5.[114] They furthermore reported that ANXA5 bound to bacterial LPS and lipid A, supporting their findings that only gram-negative, but not gram-positive, bacteria bind ANXA5, because only they have LPS in their cell wall (also see Figure 3).[68,114] To further test these observations, this present study evaluated the ANXA5 interaction with a variety of gram-negative (*Campylobacter jejuni, Neisseria gonorrhoeae*, *Escherichia coli*, *Moraxella catarrhalis*) and gram-positive (*Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis*, *Lactobacillus acidophilus* and *Lactobacillus johnsonii*) bacterial species, in addition to *H. pylori*. However, the results obtained in this study did not completely confirm the findings shown by these earlier studies (see Figure 17 and Table 3). Not only gram-negative bacteria, but also some gram-positive species (*S. aureus* and *S. pneumoniae*) bound ANXA5.

These results argue against a specific binding of ANXs to lipid A, as suggested by Rand et al., since gram-positive bacteria do not contain lipid A. Moreover, it is striking that in *N. gonorrhoeae* one strain (*N. gonorrhoeae* N356) shows the same high ANXA5 binding as *H. pylori* P12, while the other two strains (*N. gonorrhoeae* N302 and N309) bound significantly less. Additionally, all other bacterial species that were tested, and which showed a binding of ANXA5, bound significantly less ANXA5 than *H. pylori* P12. Interestingly, in this study, contrary to previously published data, certain gram-negative bacterial species (*C. jejuni* and *E. coli*) did not show an interaction with ANXA5 (see Figure 17 and Table 3). It is especially noteworthy that two bacterial species as closely related as *H. pylori* and *C. jejuni* show a different behaviour in their ANXA5 interaction.[2]

In order to investigate the reason for these differences between bacterial species and their interaction with ANXA5, some essential characteristics of the tested bacterial species were compared (see Table 5). No specific feature, such as the Gram staining or the scientific classification, shows a correlation to the ANXA5-binding behaviour or a particular similarity to *H. pylori*. It is only noticeable that all tested strains, which show an ANXA5 interaction, are human pathogens; but on the other hand not all of the tested pathogenic bacteria bound ANXA5.

Table 5 Characteristics of the different bacterial species used in this study

The table lists different features of the various bacterial strains that were tested for ANXA5 binding. Strains with ANXA5 interaction (shown in green) and without interaction (shown in red) are listed below. Strong ANXA5 binding (++), ANXA5 binding (+), no ANXA5 binding (-) (see Table 3 for details).
Source: Brock Mikrobiologie.[157]

ANXA5 Binding	Bacteria	Gram	Typ	Scientific classification		Human pathogen	Pathogenic tissue specifity
				Phylum	Class		
++	***Neisseria gonorrhoeae***	-	diplococcus	Proteobacteria	Beta Proteo-bacteria	yes	Urogenital tract + conjunctivae
++	***Helicobacter pylori***	-	rod	Proteobacteria	Epsilonproteo-bacteria	yes	stomach
+	***Staphylococcus aureus***	+	coccus	Firmicutes	Coccus	yes	respiratory tract; skin
+	***Streptococcus pneumoniae***	+	coccus	Firmicutes	Coccus	yes	respiratory tract; meninges
- +	***Moraxella catarrhalis***	+	diplococcus	Proteobacteria	Gammaproteo-bacteria	partly	respiratory tract
-	***Campylobacter jejuni***	-	rod	Proteobacteria	Epsilonproteo-bacteria	yes	Gastrointestinal tract
-	***Bacillus subtilis***	+	rod	Firmicutes	Bacilli	No	-
-	***Escherichia coli***	-	rod	Proteobacteria	Gammaproteo-bacteria	partly	Gastrointestinal and urogenital tract; meninges
-	***Lactobacillus acidophilus***	+	rod	Firmicutes	Bacilli	no	-
-	***Lactobacillus johnsonii***	+	rod	Firmicutes	Bacilli	no	-

Having confirmed that the binding of ANXA5 to *H. pylori* is most likely lipid-mediated (see above and 3.3) and taking previous studies into consideration, the interaction could either occur via phospholipids or via LPS.[88,113,114] Because the "usual" interaction partner of ANXs are phospholipids and because ANXs show different affinities to different phospholipids, it is worth to have a closer look at the membrane phospholipid composition published for the tested bacterial strains (see Table 6). ANXA5 is known to have a high affinity for phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).[88] All bacterial strains that bound ANXA5 have at least one of those phospholipids in their membrane, though some of the strains that were not interacting with ANXA5 also contain those phospholipids in their membrane (see Table 6). However, it is difficult to draw conclusions from this information as the available data about which bacterial membrane consists of which phospholipids is very inconsistent and varies considerably amongst different studies. Furthermore, a potential interaction of the bacterial phospholipids and ANXA5 is dependent on the distribution and amount of the respective phospholipid. Although PS is present in the membrane of *H. pylori*, it is considered to constitute only a minority of the phospholipids.[69] Only if the phospholipids are present in the outer membrane layer, or are flipped out upon certain triggers, they could act as potential binding partners for extracellular ANXA5. Additionally, only the interaction between *H. pylori* and ANXA5 was closely evaluated in this study and found to be most likely lipid-mediated (see 3.3). It is therefore challenging to comment on the ANXA5 binding of the other tested bacteria. Though it can most likely be excluded for *H. pylori* (see above), the ANXA5 binding of the other bacteria could still be protein-mediated and further work is needed to investigate the interaction between ANXA5 and other bacterial strains in more detail.

Table 6 Phospholipid composition of the tested bacterial strains

The phospholipids that show a high affinity to ANXA5 are phosphatidylserine (PS), phosphatidyl-ethanolamine (PE) and phosphatidylglycerol (PG) (see 1.2.1).
The table shows whether the phospholipids PS, PE and PG are present (+) or absent (-) in the membrane of the tested bacteria, or whether the available data is inconsistent (+/-) or not available (?). The strains that bound ANXA5 are listed in the upper half (ANXA5-positive), the strains that did not bind ANXA5 in the lower half of the table (ANXA5-negative).

		Phospholipids with high affinity to ANXA5			
	Bacteria	**PS**	**PE**	**PG**	*References*
ANXA5-positive	***Helicobacter pylori***	-/+ (0-4 %)	+ (12-72 %)	-/+ (0-10 %)	70,77,78
	Neisseria gonorrhoeae	-	+ (65-82 %)	+ (9-20 %)	158-160
	Staphylococcus aureus	+/-	+/-	+ (12-43 %)	161-163
	Streptococcus pneumoniae	?	?	+	164,165
	Moraxella catarrhalis	?	+ (23-38 %)	+ (33-49 %)	160
ANXA5-negative	***Campylobacter jejuni***	?	+	?	166
	Bacillus subtilis	?	?	?	163
	Escherichia coli	+	+	+	163
	Lactobacillus acidophilus	?	-	?	163
	Lactobacillus johnsonii	?	-	+	167

Because of these limitations additional work is needed. A next step for further evaluation would be to closely examine which lipid component of *H. pylori* is responsible for the ANXA5 binding. Such an examination would require performing a lipid separation by thin lipid chromatography (TLC) and performing ANXA5 overlays. Similar methods have been used before and would have to be adjusted for the specific setting.[29,113]

The interaction between ANXA5 and other bacteria was reported to be exclusive for gram-negative bacteria and mediated via bacterial LPS.[114] It is hence important to consider the possibility that the ANXA5 binding of *H. pylori* could not only take place via the bacterial phospholipids, but also with the membrane component LPS. However, these results could not be confirmed in this study, because also gram-positive bacteria bound ANXA5, and some gram-negative bacteria on the other hand did not (see Figure 17). Rand et al. used a similar procedure to the one in this study (see 2.2.1.7) with a binding assay using fluorophore-coupled ANXA5, and used a microplate spectrofluorometer instead of flow cytometry for the analysis.[114] It is therefore unlikely that methodological differences caused the observed inconsistency.

Though a variety of strains were tested both in this study and in the study of Rand et al., independent bacterial strains were used in each study. Different streptococcal species were the only bacteria, which were tested in both studies, but while *S. pneumoniae* bound ANXA5 in this study (see Figure 17), *S. pyogenes* and *S. agalactiae* did not interact with ANXA5 in the study of Rand et al.[114] It is possible that the ANXA5-binding capability of bacteria differs from strain to strain. The conclusion that the ANXA5 binding is a specific feature of gram-negative bacteria and restricted to them, as suggested by earlier studies, might have to be reevaluated, taking into account the results of this study.

LPS shows variations between different gram-negative bacteria, both in the general composition and in specific modifications.[40,168] To examine the direct interaction of LPS and ANXA5, Rand et al. used purified LPS from *P. aeruginosa* for testing the ANXA5-LPS binding.[114] Because of the varying LPS forms it cannot be concluded from these experiments that LPS from other bacteria also interact with ANXA5. On the other hand, such LPS variations could also explain why some of the tested gram-negative bacteria do not bind ANXA5 (see Figure 17), despite containing LPS in their membrane. Especially *H. pylori´s* LPS is quite unique and has a number of different modifications (see 1.1.6). *H. pylori* and *N. gonorrhoeae* both bound ANXA5 (see Figure 17) and have some LPS modifications in common. Both have a phosphoethanolamine residue at their lipid A component, though at different positions.[75,169] Moreover, both species can lack the O-side chain and produce a so-called rough LPS (see below).[170,171]

After observing variations in the ANXA5 binding of different strains of *N. gonorrhoeae* (see Figure 17), it was necessary to determine if different *H. pylori* strains showed a distinctive behaviour in their ANXA5 interaction, too. Although all tested *H. pylori* strains bound ANXA5 (see Figure 11), they showed a high variation in their binding capacity (see Figure 10). The lipid composition of the bacterial cell wall differs greatly between different strains.[70,77,78,158-160] Moreover, different strains show a great diversity in their LPS variation with loss and/or acquisition of various modifications.[7,172,173] LPS heterogeneity has also been described for different *N. gonorrhoeae* strains.[172] These dissimilarities in the lipid composition and LPS variants could explain the distinct ANXA5-binding affinities of the different bacterial strains. Of course, many other differences between strains exist, such as varying protein expression levels, virulence factors, pathogenicity and motility, that could also have influenced the varying binding capacity of the different strains.[5,6] But as the ANXA5 binding is most likely lipid-mediated (see above) it is plausible to focus on the lipid diversity of the strains.

With a lipid separation and ANXA5 overlay (as mentioned above) it could also be determined whether *H. pylori´s* LPS is directly interacting with ANXA5 and whether different wildtype strains show distinct results.

As another way to evaluate the role of LPS, a specific LPS inhibitor was used. Polymyxin B is a cationic antimicrobial peptide that is specifically working against gram-negative bacteria, because of its high affinity for LPS. Polymyxin B can “neutralize” LPS by interfering with the binding of LPS to its interaction partners.[174,175] It has been described that Polymyxin B is interacting with *H. pylori´s* LPS.[173] For other bacteria it has been shown that the blocking of LPS inhibits the ANXA5 interaction.[114] To further assess the role of *H. pylori´s* LPS in the ANXA5 binding it was therefore tested in this study whether a blocking of LPS with Polymyxin B has any effect on the ANXA5-*H. pylori* interaction. As can be seen in Figure 21, Polymyxin B treatment did not affect the binding. This could mean that *H. pylori´s* LPS is not relevant for the interaction with ANXA5 and that the binding is mediated differently, for example via phospholipids. However, there could also be various other reasons for this result: the available literature is inconsistent concerning a binding of Polymyxin B to *H. pylori´s* LPS. Though there are reports that Polymyxin B is blocking *H. pylori´s* LPS, other studies were published saying that *H. pylori* is resistant to Polymyxin B due to the unique structure of its LPS and modifications by dephosphorylation and a KDO hydrolase.[176,177] On the other hand, a study from 2006 stated that *H. pylori* cultured in serum-free medium becomes sensitive to Polymyxin B.[178] Bacteria used in this study for this experiment were cultivated without serum on cholesterol plates (see 2.2.1.1), which could mean they are susceptible for Polymyxin B. On the contrary, it has been published that cholesterol itself has no effect on the Polymyxin B sensitivity of *H. pylori*, though it contributes to LPS modi-

fications.[178,179] Considering the inconsistencies in the available literature, it is nevertheless necessary to do further experiments before drawing final conclusions on whether LPS plays a role in the ANXA5-*H.pylori* interaction or not. Anti-lipid A antibodies could be tested to block the ANX interaction, like Rand et al. did,[114] or other LPS inhibitors could be used. It would be essential to make sure in pre-tests that they work for the specific *H. pylori* strains used and specifically block LPS.

As mentioned above, cholesterol can have an influence on LPS, which is a potential binding partner of ANXA5, and was moreover described to directly influence ANXA5 binding by enhancing and stabilizing the binding of ANXs to membrane lipids.[85,179,180] In this study *H. pylori* was mainly grown on agar plates that had been complemented with a mixture of cholesterol and free fatty acids.[142] To evaluate the effect of cholesterol on the ANXA5-binding capacity, *H. pylori* cultivated on cholesterol plates was hence compared to bacteria that were grown on other agar types (complemented with serum or blood). Though a certain trend can be seen that bacteria that grew on free cholesterol supplemented plates bound the most ANXA5, the difference was not statistically significant (see Figure 22). To further investigate the effect of cholesterol *H. pylori* should be grown in cholesterol free media, as described by different authors, and then tested for its ability to bind ANXA5.[72,179]

H. pylori can incorporate host cholesterol into its membrane and modify it with the enzyme cholesterol-α-glucosyltransferase (cgt).[72] The free cholesterol, which *H. pylori* integrates into its membrane, could also have an influence on the binding of ANXA5 to the membrane phospholipids. Additionally, *cgt* mutants were shown to be slightly more sensitive to the LPS blocker Polymyxin B.[72] Because of this link between cgt and the potential ANXA5-binding partner LPS, an *H. pylori cgt* mutant was tested for its ability to bind ANXA5. However, no difference in the ANXA5 binding could be observed between the mutant and the wildtype (see Figure 23). It can therefore be concluded that cholesterol does most likely not play a role in *H. pylori´s* ANXA5 binding.

Not only did the composition of the growth media of *H. pylori* potentially influence the ANXA5 binding, but it could additionally be demonstrated that bacteria grown in liquid culture showed a markedly reduced binding of ANXA5 (see Figure 24), when compared to bacteria grown on solid agar. It has been published that both, LPS and the phospholipid composition of the membrane, which were described to interact with ANXA5, show differences whether bacteria have been cultivated in liquid or on solid cultures. It was postulated by Moran that bacteria grown in solid culture have more phosphatidylserine (PS) and lyso-Phosphatidyl-ethanolamine (PE) in their membrane than those grown in liquid culture.[76] As PS and PE are

two major binding partners of ANXA5 and could play a role in the *H. pylori*-ANXA5 interaction, this could explain the increased binding of the agar cultivated bacteria.[88]

Moreover, the LPS-forms can differ depending on the growth mode. Fresh clinical isolates of *H. pylori* possess the smooth-form LPS (S-LPS) (with a high-molecular-weight). After passaging the bacteria and cultivating them on solid agar the LPS can be modified, lose its O-side chain and be produced as rough-form LPS (R-LPS) (with a low-molecular-weight).[164] When cultured in liquid media on the other hand, *H. pylori* strains can both regain and keep the ability to produce S-LPS.[74,181] It is therefore possible that the R-LPS is capable to bind ANXA5 while the S-LPS is not. Interestingly, it was published that the LPS of a *Neisseria* strain, which showed a high ANXA5 binding comparable to *H. pylori´s* (see Figure 17), and of *P. aeruginosa*, which was reported to bind ANXA5,[114] can also lack the O-side chain.[170,171,182] The O-side chain could play an interfering role in the ANXA5 binding, for example through blocking of a major binding residue on LPS for ANXA5, and a lack of it could therefore lead to enhanced ANXA5 binding. While the major lipid A component of LPS is predominantly the same in R-LPS and S-LPS, there are still some differences.[183] Because lipid A was described as the major interaction partner of ANXA5 with other bacteria, differences here might be even more relevant for the changed ANXA5 binding than the missing O-chain.[114] In summary it can be said that both varying phospholipid composition and LPS variations could be a contributing factor to the striking reduction of the ANXA5 binding of *H. pylori* grown in liquid culture. Nevertheless, further studies are required to evaluate other differences that occur, when *H. pylori* is cultivated on either liquid or solid medium.

4.2 Annexin A5 binding and *Helicobacter pylori´s* pathogenicity

H. pylori can bind ANXA5 (see 3.1.1 and 4.1). Since certain pathogenicity factors of *H. pylori* are crucial for its successful infection of the human stomach (see 1.1.3), it was necessary to further evaluate possible consequences of the interaction with ANXA5 for *H. pylori´s* pathogenicity.

CagA is a major pathogenicity factor of *H. pylori* and an important oncoprotein for the development of gastric cancer.[27] It is injected into the host cell via the type IV secretion system (T4SS), where it causes various effects (see 1.1.3). It could be confirmed in this study that ANXA5 reduces the CagA translocation by up to ~33 % (see Figure 28), which is consistent with the data from Murata-Kamiya et al., who had previously described that ANXA5 can reduce the CagA translocation by approximately 20 %.[29] Murata-Kamiya et al. hypothesized that the reduced translocation is due to the blocking of phosphatidylserine (PS), which is needed for the injection of CagA.

They saw the same decrease in translocation when using an anti-PS antibody.[29] However, they did not investigate a possible direct interaction between ANXA5 and *H. pylori*, which was first described in this study, and did not evaluate the possibility that not only the eukaryotic cells, but also the bacteria, might play an important role in the blocking effect of ANXA5 (also see 4.4).

Because of the reduction of the CagA translocation by ANXA5 it was interesting to test whether vice versa the absence of CagA had any influence on the ANXA5 binding. It could be shown in this study that the ANXA5 binding of a Δ*cagA*-mutant is not significantly different from the wildtype (see Figure 26). The interaction of ANXA5 and *H. pylori* occurs most likely via lipid binding (see 4.1). It is therefore relatively certain that the binding does not directly occur via the CagA protein, though it could have still played a mediating role. However, it can be concluded from the experiments with the Δ*cagA*-mutant, that it most likely does not.

As a next step it was important to rule out that the reduction of the CagA translocation was a consequence of a reduced binding of bacteria to the host cells. It has not only been shown that ANXA5 is interacting with different microorganisms, but also that it can play a role for their cell binding and cell entry. It can both enhance and inhibit the interaction between different microorganisms and the host cell.[108,109] These previous findings reinforce the importance to test the effect of ANXA5 on the binding of *H. pylori* to its host cells. It could be demonstrated that the binding of the bacteria to the host cells was neither reduced nor enhanced upon ANXA5 addition (see Figure 27). These results are hence important for further investigations, as a change in the binding capacity can most likely be ruled out. This allows an evaluation of effects ”downstream” of binding steps, as the binding to the host cell serves as a first step for many pathogenic functions of *H. pylori* (see 1.1.3 and Figure 2).

Another effect of a functional Cag-T4SS and of translocated CagA is the induction of an IL-8 secretion by the host cells.[6,184] Together with other pro-inflammatory cytokines it plays an important role in the inflammation process in the stomach during an *H. pylori* infection that can ultimately lead to the development of cancer.[9,33] Because ANXA5 reduced the CagA translocation, it was relevant to see whether ANXA5 addition also showed an effect on the IL-8 secretion of AGS cells induced by an *H. pylori* infection. Interestingly, the data showed no effect of an ANXA5 addition on the amount of secreted IL-8 (see Figure 29). However, different pathways have been described for the IL-8 induction in an *H. pylori* infection. It can also occur independently of the CagA translocation, mediated by CagL, which is part of the T4SS, or via a NOD-1 activation by peptidoglycan delivered via the T4SS.[31,185,186] Up to date the exact mechanisms of IL-8 induction by *H. pylori* are still controversially discussed and remain partly unknown.[33] The reduction of the CagA translocation to two-thirds by ANXA5

might not show any effect on the IL-8 induction, because the reduction is not potent enough and other pathways could compensate for the shortage. Additionally, data from Brandt et al. suggests that CagA increases the IL-8 secretion only after an incubation of 12 hours.[187] As *H. pylori* was incubated for merely 3 hours with the cells in this study, it is possible that any effects on the IL-8 secretion caused by a change in the CagA translocation by ANXA5 might not have developed yet.

Schindele proposed the use of specific inhibitors of the CagA translocation as potential new drugs against an infection with *H. pylori*.[188] The finding that ANXA5 partly inhibits the translocation of the oncoprotein CagA opens the possibility to discuss whether ANXA5 could be used in such a way, too (see 4.5). However, this approach would be even more promising, if future studies could also show an influence of ANXA5 on the IL-8 secretion, as it is an important factor for the inflammation process. Future work should bear the longer incubation period in mind and re-evaluate the effect of ANXs on the IL-8 secretion, especially as it has been proposed that another member of the ANX family (ANXA4) has an effect on the IL-8 expression.[107]

Another major pathogenicity factor of *H. pylori* is the Vacuolating cytotoxin A (VacA) (see 1.1.3). When *H. pylori* is lacking VacA the ANXA5 binding is reduced and fewer bacteria bind ANXA5 (see Figure 26). This effect occurs independently of the presence of CagA, so in both the *vacA* and the *vacAcagA* mutant. VacA is secreted in two forms, either in a soluble or in an OMV-associated form.[36] Therefore the reduction of the ANXA5 binding could be because of a steric interference of the bound VacA form or because of an interaction between VacA and ANXA5 in the solution and a consequential blocking or clustering of ANXA5. Additionally, it has to be considered that VacA was described to interact with PS, a likely ANXA5-binding partner.[151,152] The absence of VacA might alter the lipid composition or distribution of *H. pylori´s* membrane and therefore affect the ANXA5 binding. On the other hand, the creation of a gene mutation can always affect other, unknown processes, too, and the reduced ANXA5 binding could be independent of the VacA per se. However, subsequent tests are currently performed to evaluate whether the blocking is working in both ways and if ANXA5 has any effect on the VacA-induced vacuolation of the host cells.

In summary, it could be shown that the interaction of ANXA5 with *H. pylori* can reduce the CagA-translocation *in vitro* to two-thirds, but does not show any effect on the IL-8 induction. This reduction is moreover not a consequence of an affected binding capacity of the bacteria. The ANXA5 binding is furthermore partly dependent on the presence of VacA, while it is independent of the presence of CagA.

4.3 Physiological relevance of annexins in *Helicobacter pylori* infection and carcinogenesis

ANXA5 can interact with *H. pylori* and has effects on its pathogenicity on a single cell level (see 4.1 and 4.2). As a next step the potential role of ANXs in complex infection processes was analysed to approach the physiological relevance of this novel interaction.

A chronic infection with *H. pylori* can lead to the development of gastric cancer (GC).[45,46] The bacterium is hereby the infectious agent that led to the most infection-caused tumour cases in 2012, with 89 % of non-cardia GC being attributable to an *H. pylori* infection.[47] GC causes around 700,000 deaths per year, which makes it the cancer with the third highest mortality worldwide.[189] *H. pylori* infection is therefore an extremely relevant topic with a vast social and economic impact. ANXA2 and ANXA5 are reported to be present in the human stomach and are all expressed in a variety of different cancers, including GC.[97] Different ANXs were shown to be up-regulated in GC patients and play an important role in various aspects of cancer development and progression (see 1.2.3). Lin et al. analysed gastric tissues and reported that ANXA2 and ANXA4 were up-regulated in *H. pylori*-infected stomach tumours as compared to both normal tissue and non-*H. pylori*-infected tumour tissue.[107]

Because ANXA2 is overexpressed in GC,[107] it was analysed in this study whether an *H. pylori* infection also changes the level of ANXA2 expression in GC cells. It was therefore tested whether an infection with *H. pylori* increased the amount of ANXA2 in AGS cells, a human gastric adenocarcinoma cell line. However, the data showed no significant difference in the ANXA2 expression of AGS cells before and after an infection with *H. pylori* (see Figure 31). This finding is not in accordance with Das et al., who showed an ANXA2 increase in AGS cells after an *H. pylori* infection using both mass spectrometry and Western blot analysis.[190] The incubation time they used for the infection was up to 24 hours and they stated that the maximum ANXA2 expression of the AGS cells is reached after 16 hours of infection.[190] Therefore a possible reason for the result that differed from the expectations could be that the incubation time of 3 hours used in this study might not have been enough time for the cell machinery to react and produce more ANXA2 upon the infection with *H. pylori*. Further investigations to test the ANX expression in GC cells and possible changes caused by an *H. pylori* infection should be performed, using various cell lines and looking at different ANXs, while considering to use a sufficient incubation time.

To go one step further from the cell culture experiments and to take a closer look at the physiological situation, the ANX expression was examined in human gastric tissue samples. Based on previous data, showing that ANXs are overexpressed in *H. pylori*-infected tumour tissue as compared to non-infected tumour tissue,[107] it was expected that ANXs would be up-

regulated in *H. pylori*-infected gastric tissue. To test this, immunofluorescence staining and microscopy were performed. Confirming the hypothesis, both ANXA2 and ANXA5 were up-regulated in *H. pylori*-infected gastric tissue, though the difference was only significant for ANXA5 (see Figure 32). ANXA5 can promote macrophage activation and could therefore play an important role in establishing the chronic inflammation in an *H. pylori*-infected stomach, which can ultimately lead to GC.[191]

However, it remains unclear whether the increase of ANXA2 and ANXA5 is a specific reaction to the *H. pylori* infection or a general reaction to the ongoing inflammation. ANXs have been described to play an important role in regulating inflammation processes and it would therefore be a possibility, that they are generally up-regulated in inflamed tissue.[82,191] It is moreover important to consider that the infected and non-infected gastric tissue samples were most likely from only one donor each, as they were obtained commercially. Additionally, it was not stated on the sample slides from which exact region of the stomach the samples were taken and the microscopic analysis gave the impression that they were taken from different areas of the stomach (see Figure 32). This could have falsified the results as both the ANX distribution and the *H. pylori* dissemination most likely differ between different stomach regions.[9]

For those reasons, future work is planned using tissue samples from a higher number of different patients and comparing the ANX expression of inflamed gastric tissue with and without an *H. pylori* infection. Beyond that, it is necessary to perform the data analysis with masked randomization to prevent a selection bias.

A necessary requirement for *H. pylori* to interact with ANXs in physiological context would be the presence of ANXs in the extracellular space. Different ANXs, including ANXA2 and ANXA5, occur extracellularly, though their secretion mode remains unclear.[90-92] Furthermore, ANXA2 was reported to be secreted by GC cells.[192] Using all the available data, the hypothesis was made that *H. pylori* can bind ANXA2 that is being secreted by GC cells. Tests were done to see whether an ANXA2 binding of *H. pylori* could be detected after their infection of GC cell lines (data not shown). However, up to now the assay could not be fully established. One possible explanation for this is that the amount of ANXA2 bound by the bacteria is too low for detection with the method used. Additionally, the infection time might have been too short for an ANXA2 upregulation and secretion (see above).

4.4 Consequences for the use of Annexin A5 as an early apoptosis marker in *Helicobacter pylori* research

This study is not only showing novel aspects for the infection with *H. pylori* and its pathogenicity, but also that a critical reevaluation of some methods used in bacteriological research is needed.

Independent from its physiological functions ANXA5 is used in many laboratories as a marker for early apoptotic eukaryotic cells, both *in vitro* and *in vivo*. It binds specifically to phosphatidylserine (PS), a membrane lipid which is normally present in the inner membrane, but is flipped to the outer layer of the membrane upon early stages of apoptosis (see 1.2.5).[116-121]

This study has demonstrated that ANXA5 interacts with *H. pylori* and few other bacteria (see Figure 17 and Table 3). Together with other publications that show an interaction of other microorganisms or parts of them with ANXA5,[108,109,114] this study has direct consequences for scientists working with microbes. Whenever ANXA5 labelling is used in microbiological research, the risk of cross-reactions between microorganisms (or parts of them) and ANXA5 should be considered to prevent erroneous results. Based on their research with the parasite *Leishmania*, Weingärtner et al. also proposed to be cautious when using ANXA5 as a marker for PS, as it might be unspecific.[113]

Moreover, it might be necessary to reevaluate old data and make sure the results had not been falsified by any cross-reactions. Pétillot et al. published a paper in 2007 where they used ANXA5 to detect LPS-induced apoptosis in myocardial cells in an animal sepsis model. Their results showed a significant increase in ANXA5 uptake when the rats were treated with LPS to induce sepsis.[193] However, it has been shown that the bacterial component LPS binds ANXA5.[114] Though Pétillot et al. performed further tests it remains unclear whether the ANXA5 increase was only due to the specific binding to apoptotic cells or whether the shear presence of LPS played an interfering role.

Murata-Kamiya et al. stated in 2010 that the interaction between *H. pylori* and the host cell´s PS is essential for the delivery and function of the pathogenicity factor CagA.[29] For microscopic analysis ANXA5 was used to detect PS. Here the ANXA5 signal showed an overlap with the signal of *H. pylori* (see Figure 34), which was interpreted as an externalization of PS upon infection and an accumulation at the spot of bacterial binding.[29] The outcome of this study (*H. pylori* is binding ANXA5; see Figure 8 and Figure 9) raises new questions about whether this interpretation by Murata-Kamiya et al. is correct. However, in further experiments using other methods than confocal laser scanning microscopy, the

authors could still show that there most likely is a link between *H. pylori´*s CagA and the host cell´s PS.[29]

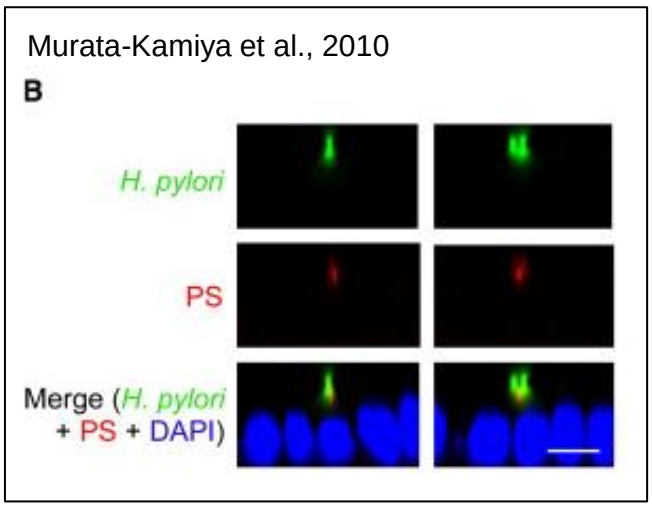


Figure 34 [Figure 1B] from Murata-Kamiya et al. showing a supposed interaction between *Helicobacter pylori* and phosphatidylserine

Figure 1B from a paper of Murata-Kamiya et al. from 2010. Confocal laser scanning microscopy of an *H. pylori* infection of eukaryotic MDCK cells. *H. pylori* (green), ANXA5 staining (red), which is claimed to be phosphatidylserine (PS), and eukaryotic nuclei (blue). Scale bar 10µm. For methodical details see Murata-Kamiya et al.[29]
Copied from Murata-Kamiya et al., Figure 1B.[29]

The novel finding of this study, that *H. pylori* binds ANXA5, is therefore extremely relevant for scientists working in *H. pylori* or other microbiological research.

4.5 Outlook: Potential medical relevance of the annexin-*Helicobacter pylori* interaction

"ANXA5 can be used as a therapeutic target with possibilities for broad applications in the early diagnosis, treatment, and prognosis of tumours in the future"[103], Peng et al. stated in 2014.[103] Many possible medical applications for ANXs have been proposed, including a therapeutic use in bacterial sepsis,[114,194-196] as a target for tumour therapy,[103,197] a preventive use as adjuvant in vaccinations,[198] and a diagnostic and prognostic use in tumours and other diseases[103,191] (also specifically for gastric cancer,[199-201] and for *H. pylori*-induced gastric cancer[107]).

As discussed above (see 4.2), other authors have suggested the use of CagA inhibitors as novel drugs in an *H. pylori* infection.[188] This study (see 3.7.2) and others could show that the CagA translocation is reduced when *H. pylori* bound ANXA5.[29] Bearing in mind that the rates of drug-resistant *H. pylori* strains are increasing, future studies should be conducted to further evaluate the *H. pylori*-ANXs interaction and to ultimately assess the potential use of ANXs as a therapeutic option in an *H. pylori* infection.[122,123]

To complete and to advance the novel findings of this study, forthcoming work should therefore:

i) Focus on the exact binding partners of ANXs by performing a lipid separation of *H. pylori* by thin lipid chromatography (TLC) and an ANXA5 overlay.[113]
ii) Further evaluate the effect of ANX binding on the IL8 induction and on other downstream effects of CagA (see 4.2).
iii) Further evaluate the effect of ANX binding on other pathogenicity factors of *H. pylori*, such as VacA, adhesion factors and the urease enzyme.
iv) Determine whether *H. pylori* can extract ANXs from the host cells.
v) Further compare ANX levels in infected and uninfected cells, gastric tissue and in gastric tumour tissue (see 4.3).
vi) Eventually perform *in vivo* experiments with analysing the addition of ANXA5 on *H. pylori* infections on the inflammation (see [114]) and with ANXA5-deficient animals.

5 Summary

5.1 Summary

Infectious diseases are a major cause of global morbidity and mortality. Approximately 50 % of the human population is infected with the bacterium *Helicobacter pylori* and many suffer from gastric pathologies caused by it. *H. pylori* is the infectious agent that leads to the most infection-caused tumour cases. Also, considering that gastric cancer has the third highest cancer mortality worldwide, *H. pylori* infections have a vast socioeconomic impact.
Annexins (ANXs) are a protein family present in the human stomach, and up to date their diverse physiological functions remain partly unknown. Previous studies have indicated both a potential role of ANXs in microbial infections as well as a possible connection with gastric cancer. The aim of this study was therefore to confirm and further characterize an interaction between *H. pylori* and ANXs and to determine possible functions and pathophysiological implications.

This study has confirmed for the first time that *H. pylori* binds ANXA1, ANXA2 and ANXA5. First, a specific binding assay was established. Following that, the study focused on the interaction of *H. pylori* with ANXA5, using flow cytometry, gel electrophoresis as well as confocal laser scanning microscopy for analysis. The binding was calcium-dependent and independent of intact bacterial proteins, which strongly suggested a lipid-mediated interaction between *H. pylori* and ANXA5 rather than a protein-mediated one. The specific role of LPS, which was previously published to be crucial in bacterial-ANX interactions, still needs to be further evaluated. *H. pylori* grown in liquid culture binds remarkably less ANXA5 than when grown on plates, suggesting a change in the lipid composition or distribution. Additionally, different *H. pylori* strains showed a different ANXA5 binding, though all tested strains bound ANXA5. Interestingly, the gram-negative bacterium *Campylobacter jejuni*, a close relative to *H. pylori*, did not bind ANXA5. Amongst the few other bacteria that also bound ANXA5, there were not only gram-negative, but also some gram-positive bacteria. This could newly be shown in this study and is contrary to previous publications, which stated that only gram-negative bacteria could bind ANXs.
CagA is one of the major pathogenicity factors and an important oncoprotein of *H. pylori* and is injected into the eukaryotic host cell. Upon binding ANXA5, the CagA translocation of *H. pylori* was significantly reduced by up to ~33 %.
A first step in understanding the relevance of ANXs in complex infection processes was to analyse human gastric tissue samples with or without an infection. It could be shown that both the ANXA2 and ANXA5 levels were up-regulated in *H. pylori*-infected gastric tissue.

An interaction between *H. pylori* and ANXs was characterized in this study for the first time. Therefore, further work is needed before the complete pathophysiology of this binding and the resulting diagnostic, therapeutic and prognostic possibilities can be assessed.

5.2 Zusammenfassung

Infektionskrankheiten tragen erheblich zur weltweiten Morbidität und Mortalität bei. Ungefähr 50 % der Weltbevölkerung sind mit dem Bakterium *Helicobacter pylori* infiziert und viele leiden unter Pathologien des Gastrointestinaltraktes, die durch das Bakterium verursacht werden. Wenn man darüber hinaus bedenkt, dass *H. pylori* den größten Anteil an infektionsbedingten Tumorerkrankungen verursacht und dass Magenkrebs weltweit die dritthöchste Mortalität aller Krebserkrankungen hat, zeigt sich die große sozioökonomische Bedeutung von *H. pylori*-Infektionen. Annexine (ANXs) sind eine Proteinfamilie, die unter anderem im menschlichen Magen vorkommt. Bis heute sind ihre vielfältigen physiologischen Funktionen noch nicht vollständig erforscht. Frühere Studien haben sowohl eine mögliche Rolle von ANXs in Infektionsprozessen als auch eine etwaige Verbindung mit Magenkrebs aufgezeigt. Das Ziel dieser Arbeit war es, eine Interaktion zwischen *H. pylori* und ANXs zu bestätigen und näher zu charakterisieren, sowie mögliche Funktionen und patho-physiologische Auswirkungen zu untersuchen.

In dieser Arbeit konnte erstmals gezeigt werden, dass *H. pylori* ANXA1, ANXA2 und ANXA5 bindet. Nachdem zuerst ein spezifischer Bindungs-Assay entwickelt wurde, wurde nachfolgend vor allem die Interaktion zwischen *H. pylori* und ANXA5 genauer untersucht, wobei Durchflusszytometrie, Gel-Elektrophorese und konfokale Laser-Scanning-Mikroskopie verwendet wurden. Die untersuchte Bindung zeigte sich sowohl Calcium-abhängig als auch unabhängig von intakten bakteriellen Proteinen. Deshalb erscheint eine Lipid-vermittelte Bindung zwischen *H. pylori* und ANXA5 deutlich wahrscheinlicher als eine Protein-vermittelte Bindung. Die genaue Rolle von LPS, welches für andere ANX-Bakterien-Verbindungen als essentiell beschrieben wurde, muss noch weiter untersucht werden. In Flüssigkultur gewachsener *H. pylori* zeigte eine deutlich verminderte Bindung an ANXA5 im Vergleich zu Bakterien, die auf Agarplatten kultiviert wurden und legten eine Veränderung der Lipidzusammensetzung oder -verteilung nahe. Des Weiteren zeigten verschiedene *H. pylori*-Stämme ein unterschiedliches ANXA5-Bindungsverhalten, wenngleich alle getesteten Stämme ANXA5 banden. Interessanterweise band das Bakterium *Campylobacter jejuni*, das in sehr enger Verwandtschaft zu *H. pylori* steht, kein ANXA5. Unter den wenigen anderen Bakterien, die ANXA5 banden, waren nicht nur gram-negative, sondern auch gram-positive Bakterien. Dies konnte erstmalig in dieser Arbeit gezeigt werden und widerspricht damit anderen Studien, die eine ANXA5-Bindung als exklusiv für gram-negative Bakterien beschrieben haben.

CagA ist ein wichtiger Pathogenitätsfaktor und ein Onkoprotein von *H. pylori*, welches in die eukaryotische Wirtszelle injiziert wird. Nach der Bindung von *H. pylori* an ANXA5 zeigte sich eine Reduktion der CagA-Translokation um bis zu 33 %.

Als erster Schritt, um die Bedeutung von ANXs in komplexen Infektionsprozessen zu verstehen, wurden Magenbiospien mit und ohne *H. pylori*-Infektion untersucht. Es konnte hierbei gezeigt werden, dass ANXA2- und ANXA5-Level in infiziertem Gewebe erhöht waren.

In dieser Arbeit konnte erstmals eine Interaktion zwischen *H. pylori* und ANXA5 beschrieben werden. Weiterführende Forschung ist jedoch notwendig, um die komplette Pathophysiologie dieser Bindung zu verstehen und die sich daraus ableitenden diagnostischen, therapeutischen und prognostischen Möglichkeiten bewerten zu können.

6 Appendix

6.1 Koch´s Postulates

1) “The organism, germ, should always be found microscopically in the bodies of animals having the disease and in that disease only; it should occur in such numbers, and be distributed in such manner as to explain the lesions of the disease.
2) The germ should be obtained from the diseased animal and grown outside the body.
3) The inoculation of these germs, in pure cultures, freed by successive transplantations from the smallest particle of matter taken from the original animal, should produce the same disease in a susceptible animal.
4) The germs should be found in the diseased areas so produced in the animal.” [15,202]

6.2 Alternative names for Annexin A5

	abbreviation
Anchorin CII	
Annexin V	
Annexin-5	
Calphobindin I	*CBP-I*
Endonexin II	
Lipocortin V	
Placental protein 4	*PP4*
Placental anticoagulant protein I	*PAP-I*
Thromboplastin inhibitor	
Vascular anticoagulant-alpha	*VAC-α*
35K-calelectrin	
35-y-calcimedin	

[83]

6.3 Abbreviations

α	Anti-
A488	Alexa Fluor® 488
A594	Alexa Fluor® 594
A647	Alexa Fluor® 647
ab	Antibody
AGS	Adenocarcinoma gastric cell line
Alexa	Alexa Fluor®
AlpA/B	Adherence-associated protein A and B
ANX	Annexin
ANXA1	Annexin A1
ANXA2	Annexin A2
ANXA5	Annexin A5
ANXs	Annexins
AnxBuf	Annexin A5 Binding Buffer
ATCC	American Type Culture Collection
BabA	Blood group antigen-binding adhesin A
BB	Brucella broth
bp	Base pair(s)
BSA	Bovine serum albumin
CagA	Cytotoxin-associated gene A
cag-PAI	Cytotoxin-associated gene A pathogenicity island
CAMPs	Cationic antimicrobial peptides
CEACAM	Carcinoembryonic antigen related cell adhesion molecules
CFU	Colony forming units
CL	Cardiolipin
CM	Complete medium; = RPMI 1640 + 10 % foetal calf serum
CMV	Cytomegalovirus
Δ	Delta (deletion)
DAPI	4′,6-Diamidino-2-phenylindole
DMSZ	German collection of Microorganisms and Cell Cultures, Leibniz Institute *Ger.: Deutsche Sammlung von Mikroorganismen und Zellkulturen*
DNA	Deoxyribonucleic acid
DPG	Cardiolipin
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ELISA	Enzyme Linked Immunosorbent Assay
EV	Enterovirus
FACS	Fluorescence-activated cell sorting; *used as abbreviation for* flow cytometer
FCS	Foetal calf serum
FI	Fluorescence intensity
GC	Gastric cancer
GFP	Green fluorescent protein
H_2O_d	Distilled water
H. pylori	*Helicobacter pylori*
Hop	*H. pylori* outer membrane protein
Hor	*H. pylori* outer membrane protein related
HPV	Human papilloma virus
i.a.	*Latin: inter alia* (=among others)
IL-8	Interleukin 8
IM	Inner membrane
kbp	Kilo base pair(s)
kDa	Kilodalton
LJ	Luisa F. Jiménez-Soto
LPS	Lipopolysaccharide
M	Molar
MALT	mucosa-associated lymphoid tissue
MDR	Multidrug resistance
MFI	Median fluorescence intensity
Min	Minutes
MOI	Multiplicity of infection
n	Number of repetitions of an experiment
n.s.	Not significant
OD	Optic density
OD_{550}	Optic density measured at the wavelength of 550 nm
OM	Outer membrane
OMV	Outer membrane vesicle
ORF	Open reading frame
PA	Phosphatidic acid
PAI	Pathogenicity island
PBS	Phosphate-buffered saline

PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEP	Peptidoglycans
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
PI	Propidium iodide
PMSF	Phenylmethylsulfonylfluorid
POX	Horseradish peroxidase
PPI	Proton pump inhibitor
PPS	Periplasmic space
PS	Phosphatidylserine
PUD	Peptic ulcer disease
PVDF	Polyvinylidene fluoride
Q	Quadrant
Ref.	Reference
R-LPS	Low-molecular-weight rough-form LPS
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium 1640
RT	Room temperature
SabA	Sialic acid-binding adhesin
SDS	Sodium dodecyl sulphate
sec	Seconds
SEM	Standard error of the mean
S-LPS	High-molecular-weight smooth-form LPS
SHP-2	Src homology 2-domains
STED	Stimulated emission depletion; *technique for high-resolution microscopy*
T4SS	Type IV secretion system
T5SS	Type V secretion system
TE	Trypsin EDTA
TLC	Thin lipid chromatography
UV	Ultraviolet
VacA	Vacuolating cytotoxin A
VBNC	Viable but non-culturable
wt	Wildtype

6.4 List of figures

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6.6 References

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6.7 Affidavit/ Eidesstattliche Versicherung

Ich, Kristina Petri, geb. am 26.07.1993 in München, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

Analysis of the interaction between *Helicobacter pylori* and members of the Annexin family

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.
Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 15.01.2020 Kristina Petri

Unterschrift

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6.9 Curriculum Vitae