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Pro-inflammatory activity, identity, and strain-specific regulation of lipopolysaccharide-derived heptose metabolites of *Helicobacter pylori*

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Summary

Bacterial heptose metabolites were reported to elicit pro-inflammatory innate immune activation by several groups including ours. The LPS intermediates are produced by diverse bacteria and were classified as MAMPs due to their stimulation of pro-inflammatory signaling via the well-established ALPK1-TIFA-NF-*κ*B axis. The Gram-negative stomach bacterium *Helicobacter pylori* produces heptose metabolites and stimulates its host in a T4SS-dependent way. While infection with the pathogen leads to an oligosymptomatic chronic gastritis, long-term infection can have serious consequences including gastric carcinoma. *H. pylori* has been associated with its human host since over 60,000 years. Therefore, the pathogen is highly adapted to its niche and even able to modulate responses of its host, securing its own survival for persistent colonization. The world-wide prevalence and the severity of disease necessitate our understanding of the pathogen's host-interaction strategies, one of them being heptose signaling. Despite extensive work by our group and others that has firmly established the knowledge about heptose signaling by *H. pylori*, many unanswered questions remained to be addressed at the beginning of this work. Thus, in my present thesis, I have investigated the effects of heptose metabolites on non-epithelial host cells, and the complex regulation and production of various heptose metabolites by *H. pylori*, including strain-specific aspects. We were able to demonstrate heptose-dependent activation of phagocytic cells, with a major contribution of the CagT4SS in its function as host-cell-directed transport system (Publication I). In the second, main part of my work (Publication II), I could show that expression of LPS heptose biosynthesis genes and pro-inflammatory host cell activation were strain-specific and dependent on regulation by an active T4SS. The expression of biosynthesis genes and metabolite production were further positively influenced upon cell contact and under control of the carbon starvation regulator A (CsrA). My results further provide strong evidence for a complex interplay of heptose biosynthesis, the CagT4SS and the central regulator CsrA that may enable upon-demand production of metabolites and feedback via the T4SS (Publication II). Further, *in vitro*-reconstitution of the biosynthesis pathway of *H. pylori* revealed various cell-active metabolites, which we identified as ADP-heptose, HBP and HMP-1 using NMR and mass spectrometry. We also detected a novel cell-active heptose monophosphate variant, likely HMP-1, in *H. pylori* lysates. Our biochemical studies uncovered additional strain-variability in heptose metabolite generation by *H. pylori* using the central enzyme HldE. Quantification of ADP-heptose in bacterial lysates revealed strong differences between various strains. These findings demonstrate the

fine-tuning of heptose-dependent inflammation and, considering heptose metabolites in a broader context, link CsrA-controlled carbohydrate metabolite biosynthesis, metabolic cross-talk, and innate immune signaling. This interplay may allow *H. pylori* to continuously balance host inflammation, ensuring a stable environment for persistent colonization. Therefore, heptose biosynthesis as a target possibly provides new perspectives for treatment or prevention of the infection.

Zusammenfassung

Verschiedene Arbeitsgruppen, darunter unsere Gruppe, konnten eine pro-inflammatorische Aktivierung des angeborenen Immunsystems durch bakterielle Heptosemetaboliten zeigen. Die LPS-Zwischenprodukte werden von verschiedenen Bakterien produziert. Heptosemetaboliten wurden aufgrund ihrer Stimulierung pro-inflammatorischer Signale über die ALPK1-TIFA-NF-*κ*B-Aktivierung als MAMPs bezeichnet. Das Gram-negative Magenbakterium *Helicobacter pylori* produziert solche Heptosemetaboliten und stimuliert seinen Wirt, auch durch die Mitwirkung eines zellgerichteten Transportsystems (T4SS). Während eine Infektion mit dem Erreger zu einer gering-symptomatischen chronischen Gastritis führt, kann eine langfristige Infektion schwerwiegende Folgen, darunter Magenkarzinome, haben. *H. pylori* migriert seit über 60.000 Jahren zusammen mit seinem menschlichen Wirt. Daher ist der Erreger in hohem Maße an seine Nische angepasst und sogar in der Lage, die Reaktionen seines Wirts zu modulieren, um für sein eigenes Überleben und persistierende Kolonisation zu sorgen. Das weltweite Auftreten von *H. pylori* Infektionen und die Schwere der resultierenden Erkrankungen erfordern, dass wir die Interaktionsstrategien des Erregers mit seinem Wirt, darunter auch die Heptose-Signalübertragung, besser verstehen. Trotz umfangreicher Arbeiten unserer Gruppe und anderer, die das Wissen über die Heptose-Signalübertragung durch *H. pylori* fest etabliert haben, verblieben vor Beginn dieser Arbeit viele offene Fragen. Daher habe ich mich in meiner vorliegenden Dissertation mit den Auswirkungen von Heptosemetaboliten auf phagozytäre (nicht epitheliale) Wirtszellen sowie mit der komplexen Regulierung und Produktion verschiedener Heptosemetaboliten durch *H. pylori* beschäftigt, was stammspezifische Aspekte einschloss. Wir konnten dabei auch bei menschlichen Phagozyten, zusätzlich zu den bisher charakterisierten Epithelzellen, eine Heptose-abhängige Aktivierung phagozytischer Zellen nachweisen, wobei die CagT4SS einen wesentlichen Beitrag als wirtszellgerichteter Transportmechanismus für Heptosen leistet (Publikation I). Im Hauptteil meiner Arbeit (Publikation II) konnte ich zeigen, dass die Expression von LPS-Heptose-Biosynthesegenen und die pro-inflammatorische Aktivierung der Wirtszellen stammspezifisch sind und auch abhängig von Variationen und Regulation durch ein aktives T4SS funktionieren. Die Expression von Biosynthesegenen und die Produktion von Metaboliten wurde durch Zellkontakt positiv beeinflusst und wird von dem Regulator CsrA mitgesteuert. Zusätzlich zeigen meine Ergebnisse ein komplexes Zusammenspiel von Heptose-Biosynthese, CagT4SS und dem zentralen Regulator CsrA, was die Produktion von Heptose-Metaboliten nach Bedarf und Feedback über das T4SS ermöglichen könnte (Publikation II). Die *in vitro*-Rekonstruktion des Heptosebiosynthesewegs

von *H. pylori* ergab außerdem verschiedene zellaktive Metaboliten, die wir mittels NMR und Massenspektrometrie als ADP-Heptose, HBP und HMP-1 identifizieren konnten. Außerdem konnten wir eine neue zellaktive Heptosemonophosphat-Variante, vermutlich HMP-1, in *H. pylori* Lysaten finden. Unsere biochemischen Untersuchungen ergaben eine zusätzliche Stammvariabilität bei der Erzeugung von Heptosemetaboliten durch *H. pylori* unter Verwendung des zentralen Enzyms HldE. Die Quantifizierung von ADP-Heptose in bakteriellen Lysaten ergab deutliche Unterschiede zwischen den verschiedenen Stämmen. Diese Ergebnisse zeigen die feine Abstimmung der Heptose-abhängigen Aktivierung und stellen, wenn man die Heptosemetaboliten in einem globaleren Kontext betrachtet, eine Verbindung zwischen CsrA, dem metabolischen Cross-Talk und dem angeborenen Immunsystem her. Dieses Zusammenspiel könnte es dem Pathogen *H. pylori* ermöglichen, so kontinuierlich die Wirtsaktivierung zu modulieren, um für ein stabiles Umfeld und eine dauerhafte Besiedlung zu sorgen. Daher bietet die Heptose-Biosynthese als Zielkomplex sehr wahrscheinlich neue Perspektiven für die Behandlung oder Vorbeugung der Infektion.

Abbreviations

ADP-hep	ADP-heptose
ADP-hep-7P	ADP-heptose-7-phosphate
ALPK1	alpha kinase 1
CsrA	carbon starvation regulator
CPC	polysaccharide capsules
DD/LD-hep	D/L-*glycero*-D-*manno*-heptose
DNA	deoxyribonucleic acid
EHEC	enteropathogenic *E. coli*
FHA	forkhead-associated domain
HBP	heptose-1,7-bisphosphate
HMP	heptose-monophosphate
IARC	International Agency for Research on Cancer
IKK	I*κ*B kinase
KDO	Keto-deoxy-octulonate
LD-hep	L-*glycero*-D-*manno*-heptose
LPS	lipopolysaccharide
MAMP	microbe-associated molecular pattern
NF-*κ*B	nucleic factor kappa B
NLR	NOD-like receptors
NMR	nuclear magnetic resonance
PAMP	pathogen associated molecular pattern
PRR	pattern recognition receptor
pT9	phosphorylated threonine position 9
RNA	ribonucleic acid
S-7-P	seduheptulose-7-phosphate
SS	secretion system
TIFA	TRAF-interacting protein with FHA
TLR	toll-like receptor
TRAF	TNF-receptor-associated factor

WHO	world health organization

1. Introduction

1.1 Bacterial heptose metabolites

Heptoses were first discovered in the 1960s as part of the bacterial lipopolysaccharide (LPS) in various bacteria (Osborn, Rosen et al. 1964, Luederitz, Risse et al. 1965, Nesbitt and Lennarz 1965, Haskins, Anacker et al. 1966, Rapin and Mayer 1966, Adams, Quadling et al. 1967). Studies on *Pasteurella multocida* for instance, identified LPS sugar components galactose, glucose and glucosamine and a heptose sugar, possibly D-*glycero*-L-*manno*-heptose (Bain and Knox 1961). LPS-focused research has taken off since 1952, when Westphal and Lüderitz introduced the hot water/phenol protocol that facilitates LPS extraction from bacterial material (Westphal, Lüderitz et al. 1952) although the concept of a toxin stemming from the cell envelope of *Vibrio cholerae* was already described in the late 19th century (Pfeiffer 1892). Later, heptoses were assigned to the inner core of the bacterial LPS (Kamio and Nikaido 1976), when the complete structure of the LPS was studied for several Gram-negative bacteria. Around the turn of the 20th century, in the wake of genetic and genomic discovery, the synthesis pathway of bacterial heptoses and the genes which encode enzymes relevant for the synthesis and heptose incorporation into the LPS were discovered (Valvano, Marolda et al. 2000, Kneidinger, Marolda et al. 2002). Bacterial heptose metabolites were then reported to be immune-activating for the first time in *Neisseria gonorrhoeae* in 2013. The study performed by Malott et al. revealed a *Neisseria*-derived heptose with a mass similar to heptose-monophosphate (HMP) in the supernatant of *N. gonorrhoeae* and marks the first evidence of induction of immune responses by bacterial heptose metabolites (Malott, Keller et al. 2013). However, two years later, the same group found evidence that the pro-inflammatory active metabolite D-*glycero*-β-D-*manno*-heptose-1,7-bisphosphate (HBP) is produced by *Neisseria* (Gaudet, Sintsova et al. 2015). Both HMP and HBP are intermediates of the LPS biosynthesis (Valvano, Marolda et al. 2000, Kneidinger, Marolda et al. 2002) and were both shown to induce immune responses in human host cells (Adekoya, Guo et al. 2018). Several groups have, in the meantime, found an immune-activating heat-stable compound, a heptose metabolite with unknown identity. Results tentatively pointed to either HMP (Malott, Keller et al. 2013), HBP (Gall, Gaudet et al. 2017, Milivojevic, Dangeard et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017), or ADP-heptose, the

third LPS intermediate first coming into the picture in 2018 (Zhou, She et al. 2018, Pfannkuch, Hurwitz et al. 2019).

1.1.1 Heptose metabolites can be found in the LPS and other structural components in various bacterial species

Originating from the inner core of the bacterial LPS (Kamio and Nikaido 1976), heptose structures can be found mostly but not solely in Gram-negative bacteria. However, there are certain exceptions of Gram-negative bacteria, which synthesize LPS as a major component of the outer membrane, but incorporate other sugars. In contrast to enterobacterial LPS, *Rhizobiaceae* possess a heptose-less core, instead using mannose (Kadrmas, Brozek et al. 1996, Di Lorenzo, Pither et al. 2020). Likewise, *Francisella* LPS inner core was reported to consist of mannose instead of heptose units (Gunn and Ernst 2007). In addition to mannose, modified glucose can be used for biosynthesis of the LPS inner core, too. Holme and colleagues studied the LPS structure of *Moraxella catarrhalis*, thereby discovering glucose phosphate as heptose substitute (Holme, Rahman et al. 1999). In *Campylobacter jejuni*, which is a Gram-negative bacterium as well, heptoses are used to build surface structures that coat the bacterial cell, so called polysaccharide capsules (CPC). CPCs are a unique feature of the intestinal pathogen, which are not associated to the LPS, thus showing that heptoses cannot solely exist as part of the LPS in Gram-negative bacteria (Guerry, Poly et al. 2012).

Besides heptose-deprived LPS and other surface structures of certain Gram-negative bacteria, examples of Gram-positive bacteria synthesizing heptose, despite not maintaining classical LPS at all, exist as well. By producing septacidin, an antibiotic with antifungal and antitumor activities, *Streptomyces* even uses the same heptose biosynthesis pathway as Gram-negative LPS producers (Tang, Guo et al. 2018).

1.1.2 Bacterial heptose metabolites were classified as MAMPs

Heptose metabolites were described in the last ten years by our group and others to act as microbe-associated molecular patterns (MAMPS, also referred to as pathogen-associated molecular patterns, short PAMPs) for various pathogens. These include *Enterobacteriaceae* like *Shigella flexneri* (Gaudet, Guo et al. 2017, Milivojevic, Dangeard et al. 2017, García-Weber, Dangeard et al. 2018), *Salmonella enterica serovar* Typhimurium (Milivojevic, Dangeard et al. 2017), *Yersinia pseudotuberculosis* and *Escherichia coli* (Zhou, She et al. 2018). Furthermore, *Neisseria meningitidis* (Gaudet, Sintsova et al. 2015, Milivojevic, Dangeard et al. 2017), *Campylobacter jejuni* (Cui, Duizer et al. 2021) and *Helicobacter pylori* (Gall, Gaudet et al. 2017, Stein, Faber

et al. 2017, Zimmermann, Pfannkuch et al. 2017) were reported to evoke immune activation heptose-dependent. Some species seem to transport heptoses through a secretion system (SS), whereas others release the metabolite directly into the surroundings. *Y. pseudotuberculosis* requires a functional T3SS (Zhou, She et al. 2018) and *H. pylori* its CagT4SS (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017) to activate pro-inflammatory signaling via heptose transport. Other bacteria which do not possess a secretion system, like *C. jejuni* and *N. meningitidis*, can activate their hosts by releasing heptoses into the environment (Zhou, She et al. 2018, Cui, Duizer et al. 2021).

The first concept of MAMPs was presented by Charles A. Janeway in 1989 (Janeway 1989, Medzhitov 2013). He proposed that MAMPs are conserved components of microorganisms, which are distinct from eukaryotic cells, thereby allowing self versus non-self discrimination by the human immune defense system. MAMPs are recognized by various germline-encoded receptors, called pattern recognition receptors (PRR), which triggers an inborn (innate) immune response (Medzhitov 2013). The recognition of MAMPs is an important feature of the innate immune system, which is one arm of the mammalian immunity working conjointly with the adaptive immune system to defend against infective pathogens. The innate immune system, in contrast to the adaptive or acquired immune system, is known as the first line of immune defense (Pasare and Medzhitov 2004). This first response is triggered by the activation of PRRs (Medzhitov 2013). Classical bacterial MAMPs are components of the bacterial cell wall such as the lipid A portion of the LPS (Poltorak, He et al. 1998), lipoteichoic acid and peptidoglycans (Takeuchi, Kawai et al. 2001), but also bacterial flagellins (Hayashi, Smith et al. 2001) or DNA (Hemmi, Takeuchi et al. 2000). These are often recognized by evolutionary very conserved receptors of the toll-like family (TLRs), or nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) leading to the production of pro-inflammatory cytokines (Medzhitov, Preston-Hurlburt et al. 1997, Girardin, Boneca et al. 2003). The discovery of the first Toll-like receptors (Toll and TLR4), interlinking developmental signaling and immune responses in various organisms, has been rewarded with two Nobel prizes to Nüsslein-Volhard in 1995 and Beutler in 2011 (Beutler 2002, Nüsslein-Volhard 2022).

Since each MAMP activates specific host PRRs and further downstream signaling, various research groups were then also determined to elucidate the signaling pathway behind heptose-dependent activation.

1.1.3 Heptose metabolites signal via the ALPK1-TIFA-NF-κB pathway

Initial evidence of LPS derived heptose metabolites stimulating host cells was found in an attempt to clarify the well-established link between gonorrhea, the sexually transmitted disease resulting from *N. gonorrhoeae* infection, and HIV-1 transmission. The study showed heptose-dependent activation of $CD4^+$ T-cells, pro-inflammatory cytokine release and HIV-1 expression in the lymphocytes in a nuclear factor κB (NF-κB) dependent manner (Malott, Keller et al. 2013). NF-κB is a family of transcriptional regulators, which mediates transcription of certain target genes upon induction by binding to a specific DNA element. NF-κB target genes are involved in pro-inflammatory and immune processes and encode chemo- and cytokines, among others (Baltimore 2009, Oeckinghaus and Ghosh 2009). However, the signal cascade initiated by heptose metabolites leading to this NF-κB activation remained unresolved at that time (Malott, Keller et al. 2013). NF-κB-dependency was later confirmed by the same group performing a genome-wide RNA interference screen (Gaudet, Sintsova et al. 2015). This study furthermore identified the gene encoding the TRAF-interacting protein with forkhead-associated domain (TIFA) to be responsible for heptose-dependent signaling and marks the first identification of a host factor involved in the heptose signaling cascade leading to NF-κB activation (Gaudet, Sintsova et al. 2015). Several studies could later verify this finding (Gall, Gaudet et al. 2017, Milivojevic, Dangeard et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). TIFA, formerly known as T2BP (Kanamori, Suzuki et al. 2002), is an oligomerizing adaptor protein which binds factor TNF-receptor-associated factor 6 (TRAF6). Oligomerization of TIFA leads to oligomerization of TRAF6, thereby activating its Ubiquitin ligase activity, leading to TRAF6 polyubiquitination and activation of the NF-κB regulator IκB kinase (IKK) (Takatsuna, Kato et al. 2003). Binding of TIFA to TRAF6 and the formation of oligomers, called TIFAsomes, is absolutely essential for activation of NF-κB (Milivojevic, Dangeard et al. 2017, Zimmermann, Pfannkuch et al. 2017). Besides the TRAF6-binding domain mediating direct TRAF6 binding, TIFA contains a phospho-threonine recognizing forkhead-associated domain (FHA), both essential for activation of IκB (Ea, Sun et al. 2004). Moreover, TIFA contains a conserved phosphorylation site (threonine 9, T9), which is bound by the TIFA-FHA domain after phosphorylation (pT9). FHA-pT9 interaction induces TIFA-TIFA self-association, resulting in TIFA oligomerization, thus stimulating NF-κB activation via IκB (Huang, Weng et al. 2012, Weng, Hsieh et al. 2015).

Alpha kinase 1 (ALPK1) was identified using RNAi as kinase responsible for the phosphorylation of TIFA at position T9 and is essential for the mediation of NF-κB activation (Milivojevic, Dangeard et al. 2017, Zimmermann, Pfannkuch et al. 2017). ALPK1 be-

longs to the family of α-kinases, is known to phosphorylate serine and threonine residues, and was first connected to the apical protein transport in epithelial cells (Ryazanov, Pavur et al. 1999, Heine, Cramm-Behrens et al. 2005, Lee, Chiang et al. 2016). The role of ALPK1 regarding heptose-dependent signaling was further revealed by Zhou and colleagues, who analyzed the crystal structure of the kinase and thereby, serendipitously, made an interesting discovery. The crystal structure of the N-terminus of ALPK1 appeared in complex with bound ADP-heptose, revealing the receptor-ligand binding mechanism of the kinase and the heptose metabolite. Thus, this study identified ALPK1 as a direct pattern recognition receptor for its ligand ADP-heptose in the heptose signaling pathway (Zhou, She et al. 2018).

Taken together, these findings describe heptose signaling via the newly identified ALPK1-TIFA- NF-κB axis, which was nicely reviewed by García-Weber and Arrieumerlou. The cascade starts with binding of the LPS intermediate metabolite to its pattern recognition receptor ALPK1, leading to the phosphorylation of threonine 9 of TIFA (pT9). pT9 is recognized by TIFA-FHA, thus forming TIFA oligomers, which leads to the formation of TRAF6 oligomers, thereby forming the so-called TIFA-somes. Activation of IκB initiates NF-κB signaling, leading to the transcription of pro-inflammatory genes. Secreted cytokines then attract immune cells to clear the infection site (García-Weber and Arrieumerlou 2021).

1.2 The model organism *Helicobacter pylori*

Many of the previous findings, by our group and others, identifying heptose metabolites as MAMPs and elucidating the heptose-dependent signaling cascade were obtained by investigating *Helicobacter pylori* infection, making *H. pylori* a good model organism to further study bacterial heptose metabolites (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017, Pfannkuch, Hurwitz et al. 2019).

1.2.1 The human stomach pathogen *Helicobacter pylori*

Helicobacter pylori is a Gram-negative human pathogen that colonizes the stomachs of half the world's population, with a higher incidence in developing countries (Malfertheiner, Camargo et al. 2023). In general, the prevalence varies within and between countries and even between different population groups (Go 2002, Suerbaum and Michetti 2002). *H. pylori* is commonly transmitted between family members, via the oral-oral or fecal-oral route of transmission, and acquisition predominantly occurs in childhood (Feldman 2001, Didelot, Nell et al. 2013, Krebes, Didelot et al. 2014).

The organism had been studied since the late 19th century (Bizzozero 1893, Salomon 1896), but research evolved further after Warren and Marshall were first able to culture *H. pylori* from patient biopsies (Warren and Marshall 1983, Marshall 2001). This achievement was groundbreaking for the investigation and treatment of stomach diseases and worth the Nobel prize in 2005. Infection with *H. pylori* always leads to a low-symptomatic chronic gastritis, but long-term infection with *H. pylori* can also have various consequences concerning the state of health, ranging from severe gastritis to duodenal and peptic ulcers, mucosal atrophy, gastric carcinoma, gastric lymphoma and MALT lymphoma (Suerbaum and Michetti 2002, Malfertheiner, Camargo et al. 2023). Several studies have shown a strong association between *H. pylori* infection and an increased risk to develop gastric cancer (Forman, Newell et al. 1991, Nomura, Stemmermann et al. 1991, Parsonnet, Friedman et al. 1991). Thus, the bacterium has been classified as type I carcinogen (a definite cause of human cancer) by the International Agency for Research on Cancer (IARC) (IARC 1994, Parsonnet 1995). However, the outcome of an *H. pylori* infection varies, between individual patients and over time, and depends, as far as we know today, on variable bacterial, host and environmental factors (Go 2002).

1.2.2 *Helicobacter pylori* and the close association and adaptation to its human host

To most microorganisms, the stomach is a rather unfavorable environment (Algood and Cover 2006). However, *H. pylori* already infected humans about 60,000 years ago and has since co-evolved with its host in the stomach (Linz, Balloux et al. 2007, Moodley, Linz et al. 2012). It is highly adapted to its niche, allowing it to colonize persistently and even life-long unless treated (Suerbaum and Josenhans 2007). This and the fact that *H. pylori* lacks a natural reservoir outside of its major host (Go 2002), emphasizes the necessity of close association of *H. pylori* to its host. Co-evolution with the human host along its world-spanning migratory routes resulted in a variety of different *H. pylori* strains of different geographical origins, which can be clustered to different populations (Linz, Balloux et al. 2007). The preferred location of *H. pylori* in the stomach is in the gastric pits, in the lower mucus layers closely associated to the surface of gastric epithelial cells (Thomsen, Gavin et al. 1990, Noach, Rolf et al. 1994, Schreiber, Konradt et al. 2004, Aguilar, Pauzuolis et al. 2022). Motility is an essential colonization factor (Eaton, Morgan et al. 1992) and is mediated by chemotaxis and spiral-shaped flagella which have been adapted to its niche (Lertsethtakarn, Ottemann et al. 2011). The capability to move in the viscous environment of the gastric mucus allows *H. pylori* to navigate towards and along the mucosal surface. To this end, *H. pylori* uses the mucus pH

gradient and chemical cues from the epithelium for chemotactic orientation (Hazell, Lee et al. 1986, Schreiber, Konradt et al. 2004, Williams, Chen et al. 2007, Rader, Wreden et al. 2011, Huang, Sweeney et al. 2015). Colonizing the stomach mucosa also has the advantage of a close to neutral pH (Schreiber, Konradt et al. 2004). In general, *H. pylori* has developed various strategies to survive persistently in the human stomach and continuously adjusts to its individual host (Suerbaum and Josenhans 2007).

1.3 Host interaction factors of *H. pylori* including heptose metabolites

The long history of co-evolution of *H. pylori* and the human host since the migration of the modern humans from East Africa (Linz, Balloux et al. 2007), coupled with the close association of the bacteria and its host in the human stomach mucosa (Thomsen, Gavin et al. 1990, Schreiber, Konradt et al. 2004), necessitate various specific modes of interaction of the bacterium with its host. The changing environment, either within the stomach of a host individual or upon new transmission, further requires constant adjustments of these bacterium-host interactions to ensure lifelong persistence (Kuipers, Israel et al. 2000, Suerbaum and Josenhans 2007, Ailloud, Didelot et al. 2019).

1.3.1 Host interaction and modulation

H. pylori modulates its interactions with the host in several ways. It is a genetically very diverse organism with a high mutation frequency (Björkholm, Sjölund et al. 2001) and the ability to exchange DNA during mixed infections (Falush, Kraft et al. 2001), allowing rapid adaptation to its general host environment and even locally to specific regions of the gastric niche (Ailloud, Didelot et al. 2019). To interact with its host, *H. pylori* can establish close contact by binding tightly to host cells via adhesins. These often highly adaptable cell-surface components facilitate adherence by binding to host structures, which is a crucial step for colonization and prevents *H. pylori* from elimination during gastric regeneration (Bonsor and Sundberg 2019). A major immunomodulator of *H. pylori* is the CagT4SS, which was acquired during evolution (Gressmann, Linz et al. 2005, Olbermann, Josenhans et al. 2010), is present in most (ca. 70%) but not all strains and is associated with high virulence (Censini, Lange et al. 1996, Mégraud 1997, Denic, Touati et al. 2020). The CagT4SS is encoded on the genetically variable *cag* pathogenicity island (*cag*PAI) together with the *H. pylori* effector protein and virulence factor CagA, which is translocated by the T4SS and injected into host cells, triggering an immune response (Censini, Lange et al. 1996, Odenbreit, Püls et al. 2000, Hatakeyama 2008). As an important driver of pro-inflammatory responses in host cells

(Guillemin, Salama et al. 2002), the functional CagT4SS is also required for host cell activation by delivered *H. pylori* peptidoglycan (Viala, Chaput et al. 2004), DNA (Varga, Shaffer et al. 2016) and heptose metabolites (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). Despite the ability to elicit pro-inflammatory responses, *H. pylori* does not appear to be sufficiently recognized and subsequently reduced or eliminated by the host immune system (Algood and Cover 2006). The prominent example of modifications of the classical MAMP LPS in *H. pylori* demonstrates the immune escape strategies of the bacterium by two major features (reviewed by Li and colleagues). Firstly, lipid A, a component of LPS, has a lower propensity to trigger immune activation via the PRR TLR4 and is resistant to host cationic antimicrobial peptides, both due to constitutive chemical modifications (Stead, Zhao et al. 2010, Cullen, Giles et al. 2011). In fact, *H. pylori* lipid A has a 1000 to 10,000-fold less endotoxicity than lipid A from *E. coli* (Birkholz, Knipp et al. 1993, Pérez-Pérez, Shepherd et al. 1995, Pachathundikandi, Lind et al. 2015). Further, *H. pylori* prevents the immune system from detecting its LPS by host mimicry of Lewis antigens at its O-antigen chains (Wang, Ge et al. 2000, Li, Liao et al. 2016). Interestingly, lipid A (Needham and Trent 2013) and the O-antigen of *H. pylori* LPS exhibit immune-evasive properties, whereas heptose metabolites, the intermediates of the inner core of LPS, elicit pro-inflammatory responses (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017), thus contributing to the pro-inflammatory arm of this particular pathogen-host interaction.

1.3.2 The LPS of *H. pylori* and the biosynthesis of heptose metabolites

As outlined above in chapter 1.1.1, bacterial heptose metabolites are produced as components for the LPS inner core (Kamio and Nikaido 1976). While most Gram-negative bacteria display this structural component of the outer membrane to maintain the barrier function and therefore a basic structure of the Gram-negative LPS has been defined, it is still very variable between, and even within, species. There are two main structural types of LPS described. The smooth type of LPS consists of lipid A, the core region and the O-antigen, while the rough LPS is partially or completely deprived of the O-antigen (Di Lorenzo, Duda et al. 2022).

The LPS structure of *H. pylori* consists of lipid A, the core oligosaccharide that is composed of inner and outer core, and the O-antigen (Altman, Chandan et al. 2011, Altman, Chandan et al. 2013). The LPS inner core of *H. pylori* consist of two L-*glycero*-D-*manno*-heptose (LD-hep) and one D-*glycero*-D-*manno*-heptose (DD-hep) residues and L-keto-deoxy-octulonate (KDO) which connects the heptoses with the lipid A part of the LPS (Li, Marceau et al. 2019). To synthesize those heptose residues, a 5-step

pathway is used, which is best characterized in *E. coli* (Kneidinger, Marolda et al. 2002). The biosynthesis cascade of *H. pylori* is similarly mediated by four partially characterized enzymes (Chang, Wang et al. 2011, Shaik, Zanotti et al. 2011, Yu, Wang et al. 2016) and produces various different heptose intermediate metabolites. The pathway starts with the transformation of the substrate seduheptulose 7-phosphate (S-7-P) to D-*glycero*-D-*manno*-heptose 7-phosphate (HMP-7) by isomerase GmhA (Yu, Wang et al. 2016). HMP-7 is phosphorylated by the bifunctional enzyme HldE, which results in D-*glycero*-β-D-*manno*-heptose-1,7-bisphosphate (HBP), followed by dephosphorylation carried out by GmhB resulting in D-*glycero*-β-D-*manno*-heptose-1-phosphate (HMP-1). The second enzymatic function of HldE transfers an ADP-moiety to HMP-1, resulting in ADP-D-*glycero*-β-D-*manno*-heptose (ADP-DD-hep) which is as the last step transformed to ADP-L-*glycero*-β-D-*manno*-heptose (ADP-LD-hep) by HldD epimerase (Chang, Wang et al. 2011, Shaik, Zanotti et al. 2011). Finally, ADP-LD-hep is transferred to KDO and lipid A by heptose transferases (Hiratsuka, Logan et al. 2005, Stead, Zhao et al. 2010). A schematic of the pathway is displayed in this work in Fig. 1 and in Publication I.

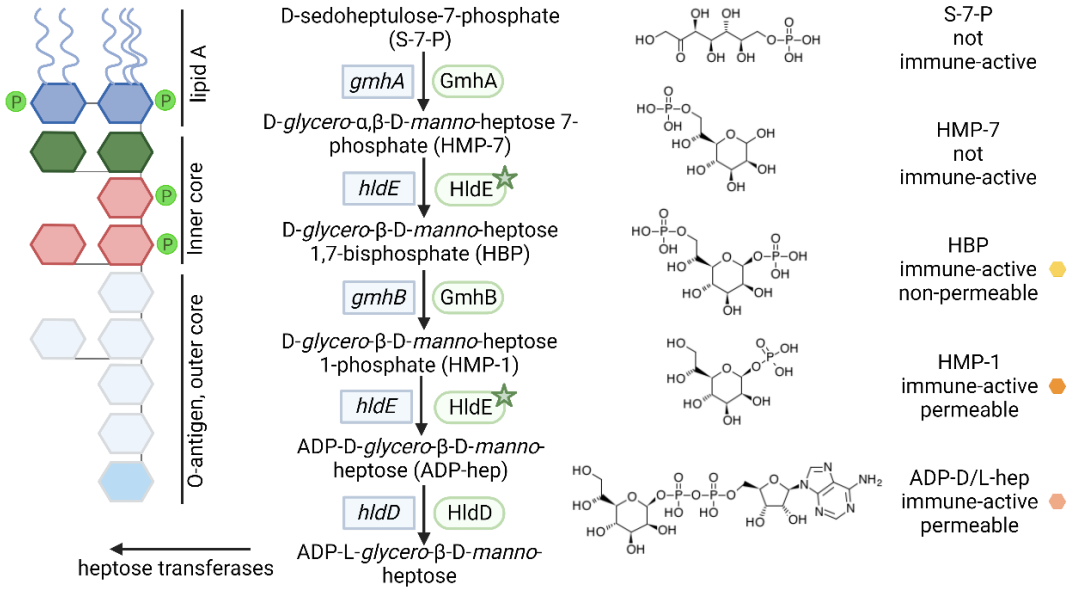

Fig. 1. Heptose biosynthesis pathway, schematic LPS, contributing enzymes and intermediate metabolites of *H. pylori*. The heptose pathway consists of five steps (as described for *E. coli* by Kneidinger and colleagues (Kneidinger, Marolda et al. 2002), conducted by four enzymes (green) that are encoded by four genes (blue). The bifunctional enzyme HldE has a highly variable sequence between different *H. pylori* strains (more details see: Stein, Faber et al. (2017) and Publication II of this thesis) and displays two known enzymatic activities, therefore catalyzing two steps of the pathway (highlighted with a star). After each step, the resulting me-

tabolite intermediates, and their structure (right side of panel) are displayed, together with their characteristics regarding pro-inflammatory immune activity and eukaryotic cell permeability (the latter according to Adekoya, Guo et al. (2018)). The end product of the pathway is transferred by heptose transferases to the inner core of the LPS, which is schematically shown on the left. The LPS structure consists of Lipid A (dark blue), inner core components (red, green), outer core (light blue) and O-antigen glycans (medium blue) (Altman, Chandan et al. 2011, Altman, Chandan et al. 2013). The LPS inner core of *H. pylori* consist of two LD-hep and one DD-hep residues (red) and KDO (green) connecting heptoses with lipid A (Li, Marceau et al. 2019). The figure was created using BioRender.com.

Four genes, clustered in one single canonical operon in *H. pylori*, encode the enzymes GmhA, HldE, GmhB and HldD, which carry out the five steps to the synthesis of LPS inner core heptose residues. The heptose biosynthesis operon is composed of genes HP0857 (*gmhA/rfaA*), HP0858 (*hldE/rfaE*), HP0859 (*hldD*) and HP0860 (*gmhB*) of reference strain 26695a (Sharma, Hoffmann et al. 2010) and is shown in this work (Fig. 1 and Publication II) and in a previous study of our group (Stein, Faber et al. 2017). The gene cluster seems to be transcribed by sigma80, a housekeeping promotor (Vanet, Marsan et al. 2000). *H. pylori* mutants deficient in heptose synthesis have growth and fitness defects, display altered LPS and are more susceptible to stress than parental strains, but are indeed viable (Chang, Wang et al. 2011, Yu, Wang et al. 2016, Stein, Faber et al. 2017). Since maintaining an intact LPS is an important housekeeping function, amino acid sequences are conserved for most, but not all, of the enzymes of the biosynthesis pathways between different *H. pylori* strains. In contrast to the other enzymes, HldE seems highly variable, shown using protein alignments in previous work (Stein, Faber et al. 2017) and this thesis (Publication II). Interestingly, the pathway also varies between species, for instance in *Neisseria*, the enzymatic activities of the bifunctional enzyme HldE are carried out by two separate enzymes (Malott, Keller et al. 2013).

1.3.3 Immune activation of host cells by *H. pylori* heptose metabolites

As described above, the heptose biosynthesis pathway yields several intermediate metabolites. Different research groups, including our group, have been working on the identification of those intermediates produced by *H. pylori* that trigger innate signaling of host cells. By mutagenesis of heptose biosynthesis genes, Stein and colleagues found that *gmhA*- and *hldE*-defective mutants were completely deficient in pro-inflammatory activation of epithelial cells, indicating that the enzymes GmhA and HldE and not GmhB and HldD are essential for heptose-dependent signaling (Stein, Faber et al. 2017). Therefore, it was proposed that HBP, the product of the activities of GmhA

and HldE, is the immunoactive metabolite (Stein, Faber et al. 2017). Zimmermann et al. also suggested this after the *hldE* mutant was unable to activate NF-*κ*B (Zimmermann, Pfannkuch et al. 2017). Results from overexpression of heptose biosynthetic enzymes in *E. coli* also pointed towards HBP (Gall, Gaudet et al. 2017). Later, Pfannkuch and colleagues made intensive efforts to verify these findings by detecting the presence of HBP in *H. pylori* lysates by mass spectrometry. To their surprise, mass spectrometry revealed only low amounts of HBP and high amounts of ADP-*glycero*-ß-D-*manno*-heptose. The fact that ADP-heptose showed high activity to induce host cell signaling led to the assumption that ADP-heptose is the predominant heptose MAMP in *H. pylori* (Pfannkuch, Hurwitz et al. 2019*)*. However, this does not necessarily exclude other heptose metabolites, especially given the strain variability of *H. pylori* (Suerbaum and Josenhans 2007). Despite the many new findings and the increasing knowledge of the biosynthesis pathway, the question of which heptose metabolites are produced by *H. pylori* and how the biosynthesis is regulated, remained unsolved.

2. Aims of the thesis

The important discovery that bacterial LPS-heptose intermediates can trigger pro-inflammatory signaling in host cells has led to a new branch of research in the field of host-pathogen interactions. Extensive work by different groups has helped to expand the knowledge of heptose signaling pathways triggered in host cells by the metabolites. Moreover, heptoses have been found in many different species, demonstrating the universal importance of heptose signaling in various host-pathogen interactions. However, many aspects of heptose metabolites were still unclear at the beginning of this work. Using *H. pylori* as a model organism, we sought to address open questions regarding the regulation of heptose biosynthesis and heptose-dependent host cell activation in *H. pylori.*

Many studies have shown that heptose metabolites trigger immune activation in epithelial cells, but little attention has been paid to heptose signaling in other types of host cells. Since there is evidence of a direct interaction between host cells of the myeloid lineage and *H. pylori* in the pathogen's niche, one aim of this work was to investigate how the heptose activation pathway contributes to the activation and maturation of human cells of the phagocytic lineage, using both macrophage-like cell lines and primary cells (Publication I).

In addition, *H. pylori* is known to modulate its host in several ways. The use of heptose metabolites to stimulate host cells is another component in the pathogen's modulation skill set. Therefore, we aimed to address the question of how *H. pylori* modulates the biosynthesis of LPS intermediates to cause heptose-dependent host cell activation (Publication II).

A variety of different *H. pylori* strains is highly adapted to their hosts and geographic origins. Since different strains have been shown to activate host cells to different extents, we wanted to determine how the pathogen regulates heptose biosynthesis and thus its activation potential in a strain-specific manner (Publication II).

Further, several studies have reported the existence of various immune-active heptose metabolites, in other species and in *H. pylori*. Thus, despite the many new findings and increasing knowledge of the biosynthetic pathway, the question of which heptose metabolites are produced by *H. pylori* remains unsolved. Therefore, an additional goal of this work was to identify potential output metabolites (products) of the heptose pathway and to compare their potential activity as novel MAMPs (Publication II).

3. Results

3.1 Publication I

Contribution of Heptose Metabolites and the cag Pathogenicity Island to the Activation of Monocytes/Macrophages by *Helicobacter pylori*.

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Contribution of Heptose Metabolites and the *cag* Pathogenicity Island to the Activation of Monocytes/Macrophages by *Helicobacter pylori*

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The human gastric pathogen *Helicobacter pylori* activates human epithelial cells by a particular combination of mechanisms, including NOD1 and ALPK1-TIFA activation. These mechanisms are characterized by a strong participation of the bacterial *cag* pathogenicity island, which forms a type IV secretion system (CagT4SS) that enables the bacteria to transport proteins and diverse bacterial metabolites, including DNA, glycans, and cell wall components, into human host cells. Building on previous findings, we sought to determine the contribution of lipopolysaccharide inner core heptose metabolites (ADP-heptose) in the activation of human phagocytic cells by *H. pylori*. Using human monocyte/macrophage-like Thp-1 cells and human primary monocytes and macrophages, we were able to determine that a substantial part of early phagocytic cell activation, including NF-κB activation and IL-8 production, by live *H. pylori* is triggered by bacterial heptose metabolites. This effect was very pronounced in Thp-1 cells exposed to bacterial purified lysates or pure ADP-heptose, in the absence of other bacterial MAMPs, and was significantly reduced upon TIFA knock-down. Pure ADP-heptose on its own was able to strongly activate Thp-1 cells and human primary monocytes/macrophages. Comprehensive transcriptome analysis of Thp-1 cells co-incubated with live *H. pylori* or pure ADP-heptose confirmed a signature of ADP-heptose-dependent transcript activation in monocyte/macrophages. Bacterial enzyme-treated lysates (ETL) and pure ADP-heptose–dependent activation differentiated monocytes into macrophages of predominantly M1 type. In Thp-1 cells, the active CagT4SS was less required for the heptose-induced proinflammatory response than in epithelial cells, while active heptose biosynthesis or pure ADP-heptose was required and sufficient for their early innate response and NF-κB activation. The present data suggest that early activation and maturation of incoming and resident phagocytic cells (monocytes, macrophages) in the

H. pylori–colonized stomach strongly depend on bacterial LPS inner core heptose metabolites, also with a significant contribution of an active CagT4SS.

Keywords: *Helicobacter pylori*, Heptose, macrophage, secretion system, type four secretion system, heptose biosynthesis pathway, lipopolysaccharide

INTRODUCTION

The bacterium *Helicobacter pylori* is a chronic human pathogen of major global importance, since about half of the world population carry this bacterial species in their stomach (1). *H. pylori* colonizes primarily the gastric crypts and interacts with the gastric epithelial cell layer. The interaction of *H. pylori* with epithelial cells, primarily of gastric origin, has been very well studied in the past. It is known that *H. pylori* adheres to gastric epithelial cells very specifically, using several different bacterial surface adhesins (bacterial outer membrane proteins), such as AlpA/B, SabA, and BabA/B, HopQ (2–5). On the host (human) side, various receptors, including Lewis antigens (3), cellular integrins, and carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (6–9) are involved in the interaction. *H. pylori* activates gastric epithelial cells by different innate immune pathways upon intimate cell adherence, involving, among others, TLR2 (10), TLR9 (11, 12), NOD1 (13), and NLRP3 (14, 15) receptors and the newly described ALPK1-TIFA dependent pathway (16–18), which lead to the activation of diverse downstream proinflammatory signaling pathways (19, 20). The ALPK1 pathway is activated mainly by bacterial inner core lipopolysaccharide heptose metabolites, heptose-1,7-bisphosphate (HBP) and, predominantly, ADP-glycero-ß-D-manno heptose (ADP-heptose), which have been reported to interact directly with the kinase ALPK1, resulting in TIFAsome formation and NF-κB activation (18, 21, 22). TLR4 and TLR5 cellular receptors, widely present on epithelial cells, appear to be less activated by the bacteria, since the respective bacterial surface molecules (microbe-associated molecular patterns [MAMP]) of *H. pylori*, lipopolysaccharide (LPS/lipid A) and flagellins, have evolved to low activation potential (10, 23–26). Another very well-studied cell interaction module of *H. pylori* with respect to gastric epithelial cells is the *cag* pathogenicity island (*cag*PAI). The presence of the *cag*PAI determines the extent of gastric inflammation and subsequent disease severity in patients (27) and in animal models (28). This large genetic element is located on the *H. pylori* genome in about 70% of all global isolates (29) and encodes a membrane-spanning secretion system of the type IV (CagT4SS) (30–32). The Cag secretion system is expressed by *H. pylori* in the stomach (33, 34) and can translocate various small molecules into gastric epithelial cells, including bacterial DNA (12), the NOD1 innate receptor ligand ieDAP (13) and lipopolysaccharide (LPS) heptose precursors, HBP and ADP-heptose (17, 21). These small molecule metabolites contribute to different extents and at different times to the epithelial cell activation and modulation by *H. pylori* (13, 16, 20, 35, 36). A substantial portion of early NF-κB activation of epithelial cells by *cag*PAI-positive *H. pylori* appears to be mediated by the CagT4SS-mediated transport of inner core heptose metabolites into the gastric epithelial cells (16–18). At least two CagT4SS transported molecules, the oncogene CagA (37–40) and the peptidoglycan metabolite ieDAP (13, 16), provide signals of sustained, late cell activation (16, 41).

Little information is as yet available on the precise molecular mechanisms of interaction and crosstalk of *H. pylori* with human cells of the myeloid lineages, for instance the phagocytic and antigen-presenting cells (14). The colonization niche of *H. pylori* deep in the gastric mucus layer and within the gastric crypts is characterized by the continuous presence and permanent low-level influx of cells of the myeloid and lymphatic lineages. The immigration of phagocytic cell types, for instance, macrophages and neutrophils, is increased during *H. pylori*-induced inflammation (42). The myeloid and lymphoid cell lineages that *H. pylori* can contact in the gastric mucosa comprise antigen-presenting cell types such as macrophages or dendritic cells, which are probing the mucosa-adherent bacteria or migrating into the mucosa in response to inflammation and cytokines. Neutrophils are attracted to the site of infection, once *H. pylori* have activated local proinflammatory signaling, leading to the secretion of chemokines such as IL-8 (42, 43). It is known that NLRP3 plays a role both for IL-1β production by phagocytic cells in response to *H. pylori* and for bacterial suppression by the immune system *in vivo* (14, 15). The bacterial *cag*PAI is known to be important for the interaction of macrophages with *H. pylori*, even in the absence of TLR signaling (44). Furthermore, it was reported that *H. pylori* directly interacts with stem cells in the depth of gastric crypts (45, 46).

On the basis of the recent discovery of novel cell activation pathways by *H. pylori*, involving the intracellular activation of the ALPK1-TIFA axis and the formation of TIFAsomes, mediated by the inner core LPS heptose metabolite ADP-heptose (16–18, 21), we have now asked the question, how this specific cellular activation pathway contributes to the early and sustained activation and maturation of human cells of the phagocytic lineage, using the human monocyte-like cell line Thp-1 (47, 48) as a model. We demonstrate here that the *cag*PAI-dependent heptose metabolite-triggered early innate activation contributes to a significant extent to cell activation and maturation of human Thp-1 cells and also activates primary human monocytes and monocyte-derived primary macrophages. We also underline and broaden these results by comprehensive transcriptome analyses of heptose- or bacteria-co-incubated Thp-1 cells.

MATERIALS AND METHODS

Bacterial Strains and Cultivation

H. pylori bacteria of different strains and mutants (**Table 1**) were cultured under microaerobic conditions (Anaerocult C

TABLE 1 | Bacterial strains and description.

Strain name	Origin	Description	Reference
H. pylori (HP) N6 wt	hpEurope; site of isolation = France	Wild type strain, *cag*PAI-positive	(49)
HP N6 *cagY* (HP0527)	hpEurope	Allelic exchange-insertion mutant (HP0527) *(cagY)* in strain N6	(17)
HP N6 858 (HP0858) *(hldE)*	hpEurope	Allelic exchange-insertion mutant (HP0858) (*hldE*) in strain N6	(17)
HP N6 858 (HP0858) comp	hpEurope	HP0858 gene complementation in *rdxA* locus of HP0858 knockout strain N6	(17)
HP 26695a (88-3887) wt	hpEurope; site of isolation = USA	Wild type strains, *cag*PAI-positive	(50)
HP 26695a Δ*cag*PAI	hpEurope	*cag*PAI complete deletion by allelic exchange in strain HP 88-3887 (plasmid pCJ324)	(17)
HP 26695a *cagA*	hpEurope	*cagA* allelic exchange insertion mutant in strains HP 88-3887	(17)
HP L7 wt	hpAsia2, South Asia, India	Wild type strain, *cag*PAI-positive	(29)
HP J99 wt	hpAfrica1, North America	Wild type strain, *cag*PAI-positive	(51, 52)
HP TAI196 wt	hpEastAsia	Wild type strain, *cag*PAI-positive	(29)
HP Africa2 wt	Africa2	Wild type strain, primary *cag*PAI-negative	(51)
HP Su2 wt	hpNEAfrica, Northeast Africa, Sudan	Wild type strain, *cag*PAI-positive	(29)

sachets by Merck) on blood agar plates (Oxoid Blood Agar Base No.2) including 10% horse blood (Oxoid) and the antibiotics (all purchased from SIGMA) amphotericin B (4 mg/L), polymyxin B (2,500 U/L), vancomycin (10 mg/L), trimethoprim (5 mg/L), chloramphenicol (optional; 5 mg/L), and kanamycin (optional; 10 mg/L). Brain Heart Infusion broth (BHI, Becton-Dickinson) supplemented with 5% horse serum (Thermo Fisher Scientific-Gibco) was used for liquid culture of *H. pylori* strains. Routinely, bacteria were cultured at 37°C for 20 h to 24 h on blood agar plates in anaerobic jars, supplemented with humidified Merck Anaerocult C sachets, or in incubators under microaerobic atmosphere (10% CO_2, 5% oxygen, 85% nitrogen) and passed to fresh plates at least on every second day. Liquid cultures were incubated under microaerobic atmosphere with shaking until mid-log phase (OD_{600} between 0.5 and 1.0), for the collection of culture supernatants. Enzymatically treated lysates (ETLs), enriched in metabolites, were prepared as in (17).

Cell Lines and Culture Conditions

In this study, we used the human monocyte leukemia cell line Thp-1 (ATCC TIB202 (47, 48). Thp-1 cells are widely used as a model for monocyte-macrophage-like cells and can be differentiated into active macrophages, for instance using phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) treatment (47). For priming, we applied PMA (50 ng/well (53); for 2 days to Thp-1 cells, then gave them a one-day resting period, and then co-incubated the cells with the bacteria on the fourth day, for 4 h. In addition, we employed the NF-κB reporter cell line Thp1_luc (kindly provided by Karsten Tedin) for quantitating NF-κB-dependent responses, and the NLRP3-deficient Thp-1 cell line derivative Thp-1 dNLRP3def (Invivogen). All cell lines were cultured in RPMI1640 medium (buffered with 20 mM Hepes, Glutamax; Thermo Fisher Scientific - Gibco) supplemented with 10% FCS (PromoCell) and, in the case of Thp1_luc cells, were additionally supplied with 0.5 µg/ml puromycin (except when co-incubated with live bacteria or bacterial products). Cells were routinely cultured in a 5% CO_2 atmosphere incubator. Co-incubation times for each experimental setting are indicated in the figure captions and in the results.

Co-Culture of Cells With Live Bacteria or Bacterial Products

Co-incubation experiments cells with Thp-1 cells were conducted in either 96-, 24- or 6-well cell culture plates (Greiner BioOne). Thp-1 cells were seeded into fresh RPMI1640 medium 1 h prior to co-incubation with bacteria. Exponentially growing *H. pylori* were harvested from plates and, *via* OD_{600} quantitation, adjusted to the respective multiplicities of infection (MOI) of live bacteria (5, 10, or 25, as detailed for each experiment in the results and respective figures) in RPMI1640 medium containing 10% FCS. Subsequently, macrophages were co-incubated with various bacterial strains, and cell interaction was synchronized using centrifugation (300 × g, 5 min at RT). We chose different co-incubation periods for each type of experiment: For the NF-κB luciferase reporter cell assay, co-incubation was carried out for 4 h. In the case of intended RNA-isolation, cells were co-incubated with bacteria or bacterial products for 8 h. For ELISA cytokine measurements, co-incubations were conducted for 20 to 22 h, before we harvested the cleared cell culture supernatants. Mock-infected cells were used as negative control in each experiment, and Pam3Cys-SK4 (PAMCys, a canonical TLR2 ligand; stock solution of 20 ng/µl) (Invivogen), adjusted to cell number and medium volume in each well plate (standard dilution of 20 ng/50 µl of medium), was co-incubated with cells in each experiment as positive control. In case of comparisons between experiments of the same type, PAMCys coincubation was also used as a normalization control. For further analysis of co-incubated cells *via* RNA-based methods or ELISA, cell pellets and supernatants were harvested and the latter cleared by centrifugation (13,000 × g, 2 min, RT).

Cells were co-incubated with bacterial enzyme-treated lysates (ETLs) at different amounts (volumes between 1 and 50 µl), normalized to bacterial numbers and culture volumes, prepared as outlined in (17). For 96-well co-incubations, we usually used 2.5 µl of bacterial ETL per well (50 µl volume). In 24-well plates, 50 µl of bacterial ETL per 1 ml culture volume were added. By titration in Thp_luc cells, we estimated that 2.5 µl of ETL contained approximately 2.5 µM active heptose metabolites, since the activation was equivalent. Cells were also challenged with pure ADP-heptose (J&K, China) and Pam3Cys-SK4 (Invivogen; activation positive control and normalization control) at concentrations indicated in the results text and

figure legends, respectively. To control for cell death, e.g. by pyroptosis, cells during the co-incubation period of 20 h were subjected to a fluorescent live-dead cytotoxicity assay according to the manufacturer's instructions (CellTiter-Blue Cell Viability Assay; Promega). The results for the different control and co-incubation conditions (bacteria, ADP-heptose) did not show significant signs of cell-death and were not different from the mock co-incubated control condition.

Cytokine Measurements

We quantitated the following cytokines released into cell supernatants using ELISA measurements: human IL-8 (BD OptEIA set #555244), human IL-1β (BD OptEIA set #557953), human IL-10 (DuoSet ELISA R&D #DY217B-05), human IL-6 (BD OptEIA set #555220), human CCL4 (DuoSet ELISA R&D Systems #DY271-05), human IFN-γ (BD OptEIA set #555142). Cytokine secretion into cell supernatants was quantitated using the above commercial ELISA sets and included standard dilutions according to the manufacturer's instructions. Appropriate dilutions of the cell supernatants for each assay were determined by suitable pre-testing.

Luciferase Quantitation in Reporter Cells

To quantitate luciferase in NF-κB reporter cells, the Steady-Glo Luciferase Assay System (Promega) was used. Briefly, the lysis buffer-substrate mixture of the system was added to each well and the lysis was allowed to proceed as recommended by the manufacturer's instructions [see also (54)]. Lysed cells were analyzed for photon counts within 10 min after the lysis using a Clariostar multi-well reader (BMG Labtech) in luminescence mode (acquisition of photons for 10 s, no filter); output is quantitated as counts per second.

RNA Isolation, cDNA Synthesis and Transcriptome Sequencing (RNA-Seq)

RNA was prepared from Thp-1 cells (monocyte/macrophages) grown in 6-well plates (ca. 2×10^6 cells per well) under co-incubation conditions with *H. pylori* N6 wild type and N6 858 *hldE* mutant (both co-incubated at MOI = 10) and with pure ADP-heptose (at a concentration of 5 μM) for 8 h.

In brief, cell pellets were collected after scraping and rinsing the cells from the plates by centrifugation at 22,000 × g, 1 min, at room temperature. Cell pellets were shock-frozen in liquid nitrogen and stored at −80°C. Total RNA was prepared from each cell pellet using a modified RNeasy spin column protocol (Qiagen, Hilden, Germany) after mechanical lysis in a Fastprep bead-beater (MP Biomedicals Inc., Santa Ana, CA, USA), at power setting 6 for 45 sec. The isolated RNA was quantitated and quality-assessed by photometric measurement, on agarose gels and by tape station (Agilent, Tape Station 4200, RNA nano kit, Agilent) quality controls. All RNAs were treated once with DNaseI (TURBO RNAse-free DNA removal kit; Ambion) according to the manufacturer's protocol. PCR controls for human GAPDH were performed on each RNA sample pre- and postDNAse treatment to clarify that residual DNA had been removed successfully.

cDNA was routinely synthesized from 1 μg of total RNA, using a combination of random hexamer primers and oligo-dT T12-T18 primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen) at 42°C for 2 h.

Transcriptome sequencing and analysis was performed after careful quality control of each sample using Agilent tape station and Agilent Bioanalyzer, from 1 μg of total RNA of each sample, using the Illumina NextSeq 500 platform and enrichment of messenger RNAs using poly-T primers before library preparation (non-stranded libraries). The sequencing process was set for a read length of 50 bp on average in each cycle. The output primary raw reads were quality-filtered and trimmed (removal of primer sequences and barcoding) before further analysis.

Bioinformatics Analysis of RNA-Seq Data

Un-paired fastq files containing trimmed and quality-filtered reads of 50 bp on average for each experimental condition were collected from the sequencing platform (Illumina). For further transcriptome analysis, quality-filtered, trimmed reads were further processed *via* the CLC Genomics Workbench Version 10.1.1 or a higher version (QIAGEN Aarhus A/S). The sample size for all samples was defined to be an equal 2,000,000 reads, and all samples were downsampled (reproducible random setting for downsampling) to this read number. Subsequently, all remaining reads after downsampling were mapped against the human reference genome hg18 (*Homo sapiens* reference genome, ncbi database) in gene track mode (no transcript variants were recorded or analyzed). Read alignment settings were as follows (default settings):

- Mismatch cost = 2
- Insertion cost = 3
- Deletion cost = 3
- Length fraction = 0.8
- Similarity fraction = 0.8
- Strand specific = both
- Maximum number of hits for a read = 10

Expression level options in the RNA-Seq module of CLC Genomics Workbench were set as follows: expression value = Total count; calculate RPKM for genes without considering differential transcripts. Quality control parameters from the sequencing and genome mapping were recorded and compared for all samples according to (**Table 2**). All samples displayed uniform and high-quality control parameters.

RPKM (reads per kilo base [gene length] per million [read count]) values were used for the expression level analysis and further comparisons between samples, as implemented in CLC Genomics Workbench. Differential expressions of all conditions were calculated for all conditions with mock samples as the reference condition, which served as control group. For Venn diagram generation of comparisons between conditions, differential expression values with an absolute fold-change higher than two, four or eight, as indicated in

TABLE 2 | Quality control parameters of comprehensive transcriptome sequencing (RNA-Seq).

Parameters		(N) Thp-1 mock	(O) Thp-1 + N6 wt MOI10	(Q) Thp-1 + N6 858 MOI10	(Z) Thp-1 mock	(A1) Thp-1 + ADP-heptose 5 µM	(A2) Thp-1 mock	(B2) Thp-1 + ADP-heptose 5 µM
Mapping statistics	Reads mapped (%)	81.85	81.88	80.87	83.99	82.23	82.55	82.03
	Reads not mapped (%)	18.15	18.12	19.13	16.01	17.77	17.45	17.97
Fragment counting	Unique (%)	79.12	79.25	78.1	81.47	79.52	79.83	79.27
	Non-specifically (%)	2.73	2.61	2.77	2.52	2.71	2.72	2.76
Counted fragments by type	Intergenic (%)	11.17	10.79	11.65	9.56	10.26	7.72	8.03

the respective figures and tables, were taken into consideration. The full results of differentially expressed transcripts are shown in **Supplementary Tables S1** and **S2**. The complete transcriptome results are accessible under project no. PRJNA685657 at ncbi.

For protein network analysis from the resulting transcriptome data, the STRING database [https://string–db.org/, (55)] was used. Highly differentially expressed genes overlapping between different samples (threshold of two-fold, four-fold or eight-fold regulated) were uploaded into the platform. Network analysis was performed with a minimum required interaction score of medium confidence (0.004), and only query proteins were allowed to be shown as maximum number of interactions. An absolute fold-change of four-fold or eight-fold regulated transcripts (up- or down-regulated) was used as a threshold to provide a selection of regulated transcripts for further analysis and visualization in STRING as shown in the figures (**Figure 3**; **Supplementary Figure S6**; ball diagrams).

The following STRING settings were used: Proteins with Values/Ranks: minimum required interaction score: medium confidence (0.004); maximum number of interactions to show: 1st shell: none/query proteins only; 2nd shell: none. The level of confidence and the maximum number of interacting proteins were set at 0.4 and 5, respectively.

Quantitative Real-Time PCR

We performed quantitative (q)RT-PCR as described previously (17). Briefly, (q)RT-PCR was performed on pretested amounts of cDNA specific for each transcript (between 0.5 and 2.5 µl per reaction) using gene-specific primer pairs for human genes (Qiagen Quantitect primer set; primers see **Table 3**), ultrapure water and SYBR Green Master Mix (Qiagen). We performed the reactions in a BioRad CFX96 real-time PCR system (BioRad, Hercules, USA). Quantitation of specific mRNA transcripts in the samples was carried out in technical triplicates. Standards for quantitation in each run stemmed from gene-specific PCR products amplified with the same primers. Results were equalized to 0.5 µl cDNA input and normalized to human GAPDH transcript of each condition, using the Hs_GAPDH transcript amounts from mock-co-incubated cells as reference condition. The MiQE quality settings applied for each qPCR were as described in (56).

TABLE 3 | qPCR primers for human genes (Quantitect or RT2—Qiagen).

Name	Gene Symbol	Cat. No
Hs_CXCL8_1_SG	CXCL8	QT00000322
Hs_IL1B_1_SG	IL1B	QT00021385
Hs_ALPK1_1_SG	ALPK1	QT00100842
Hs_CCL4_1_SG	CCL4	QT01008070
Hs_TLR2_1_SG	TLR2	QT00236131
Hs_NLRP3_2_SG	NLRP3	QT01666343
Hs_GAPDH_2_SG	GAPDH	QT01192646
Hs_NOD1_1_SG	NOD1	QT00054082
Hs_TIFA_1_SG	TIFA	QT00212779
Hs_IRF3_1_SG	IRF3	QT00012866
Hs_IRF7 (RT2)	IRF7	PPH02014F (RT2)

Isolation of Primary Human PBMCs and CD14+ Cells

Human PBMCs were isolated from human blood using negative depletion *via* the MACsPrep PBMC Isolation Kit (negative selection) from 40 ml of whole, serum-depleted anticoagulated blood from healthy donors obtained anonymously from a commercial blood bank. For the isolation of primary $CD14^+$ blood monocytes from PBMCs or directly from whole blood/plasma apheresis specimens of anonymous healthy human donors (blood bank), human CD14+ beads (Miltenyi Biotech, Germany), were used in a positive selection protocol as described in the manufacturer's manual. Alternatively, the selection of primary monocytes from PBMC by plate adherence protocol for monocyte enrichment was used according to (57). After isolation, the monocytes were rested for 24 h in fresh RPMI 1640 supplemented with penicillin/streptomycin and 10% FCS, and then co-incubated with pure ADP-heptose for 20 h at various concentrations indicated in the figure legend. Alternatively, CD14+ monocytes were differentiated with macrophage-colony-stimulating factor (human M-CSF; Peprotech, Germany) for seven days, with one medium change in between. At the end of the differentiation period, cells were visually inspected to be positive for phenotypic maturation, well adherence and colony formation, the medium was changed again to fresh medium (without penicillin-streptomycin and M-CSF) and cells were co-incubated with pure ADP-heptose, PAMCys (positive control for activation), live *H. pylori* bacteria or bacterial treated lysates (ETLs), as indicated in the figure legend, for 4 h. Subsequently to the co-incubations, cell supernatants were collected for cytokine measurements, and cell pellets were collected for RNA isolation. Monocyte purity was verified as follows: collected

monocytes were washed once with PBS and resuspended in staining solution (50 µl), consisting of FACS buffer (PBS, 1% FBS, 2 mM EDTA) and α-CD14 Ab (PE clone: M5E2 BD; 555398), and incubated for 20 min at 4°C. Cells were again washed and resuspended in 100 µl FACS buffer. Finally, stained cell suspensions were analyzed in a BD FACS Lyric (Becton, Dickinson and Company).

siRNA Knock-Down in Thp-1 Cells

For siRNA knock-down of human TIFA transcript in Thp-1 cells, we used the Flexitube Qiagen siRNA Assays, containing four different validated siRNA variants against human TIFA (Flexitube GeneSolution: Hs_TIFA-6, Hs_TIFA-7, Hs_TIFA-8, Hs_TIFA-9, each at 10 µM stock concentration), according to the manufacturer's instructions. Thp-1 cells (2×10^5 per well) were transfected with siRNAs using the LONZA nucleofector kit SG (nucleofector 4D), with 1.25 µM of siRNA Mix for each siRNA per well (5 µM total of siRNA per well if all were combined, in strip well transfection cuvettes). Immediately after nucleofection, all cells from each cuvette well were resuspended in 1 ml of fresh medium (RPMI1640, 10% FCS) per well, each seeded in 24 well plates, and let acclimatize for 72 h. For each experiment, transfection mix for mock transfection (transfection agent without RNA) and Allstars Negative Control siRNAs Mix (Qiagen) were transfected in separate wells alongside, as negative controls for the knock-down. After 72 h, the cells were again incubated in fresh medium and subsequently incubated in the presence of pure ADP-heptose (5 µM per well), as indicated in the figure captions. At the end of the co-incubation period, cells were gently scraped off the wells, collected including their supernatants, which were then harvested by centrifugation for cytokine ELISA. Cells recovered in the pellets by centrifugation from the supernatants were immediately resuspended in RNA-Later (Agilent) for undamaged RNA isolation. RNA was subsequently isolated from each cell pellet as described above, quality-tested, and used to verify the specific knock-down by qRT-PCR for TIFA transcript (normalized in each condition to Hs_GAPDH transcript). For luciferase detection after siRNA transfection, Thp1_luc cells (2×10^5), containing the NF-κB luciferase reporter, were transfected with siRNA or mock as described above in one strip well, then each transfection well was distributed further into 5 wells of a 96-well plate, in 100 µl of medium (final cell count per well of ca. 4×10^4). The cells were again kept for 72 h for expressing the siRNA. Subsequently, medium was changed to 50 µl fresh RPMI, the cells were co-incubated with pure ADP-heptose for 4 h and then subjected to lysis and luciferase measurement (Promega Steady-Glo firefly luciferase substrate).

RESULTS

Live *H. pylori* Activates Thp-1 Monocyte/Macrophage Cells, Dependent on Active Heptose Biosynthesis

On the basis of previous results using human epithelial cells (17, 18) we wanted to verify, whether live *H. pylori* bacteria activate phagocyte-like cells using similar pathways or metabolites. We used Thp-1 cells as a model. Thp-1 is a human monocyte leukemia cell line that differentiates into macrophages upon phorbol-12-myristate-13-acetate (PMA) treatment (47). Thp-1 cells have been used extensively to study monocyte/macrophage signaling pathways, responses and phagocytosis mechanisms (47, 58). In these assays, we also wanted to assess at the same time, whether the bacteria engage predominantly TLR-mediated (15, 25, 26), NLR-mediated (13–15), or (ADP-)heptose metabolite-mediated recognition (17, 21, 22), in order to activate phagocytic cells at early time points. We initially co-incubated Thp-1 cells with live *H. pylori* bacteria for different time periods. We used different *H. pylori* strains and mutants, including mutants deficient in the CagT4SS and mutants deficient in the LPS-heptose biosynthesis pathway (17) for the cell activation. Thp-1 monocyte-like cells were alternatively pretreated (primed with PMA) to differentiate them into an adherent, macrophage-like cell type, or not pretreated, before co-incubating them with live *H. pylori*. IL-8 cytokine release into the cell supernatant was quantitated, as an outcome of either IL-1/TLR receptor pathway activation or as a marker for LPS core heptose activation in the cells. In addition, IL-1β secretion by the cells and transcript amounts were determined, as IL-1β is a signature cytokine for combined IL-1/TLR and NLR pathway and inflammasome activation. When we co-cultured non-primed Thp-1 cells with live *H. pylori* bacteria for at least 8 h (20 h time point shown (**Figure 1A**), the cells started to morphologically differentiate into an adherent cell type, corresponding to a macrophage-like cell morphology. IL-8 cytokine secretion was determined by ELISA. The amount of IL-8 released at 20 h post-co-incubation was partially associated with an active *cag*PAI and strongly correlated with active LPS heptose biosynthesis (**Figure 1**). Cell activation by heptose (*hldE*) mutants in this setting was significantly lower than by parental wild type bacteria, but significantly higher than for mock-co-incubated cells. Likewise, IL-1β release upon *H. pylori* co-culture was influenced by heptose biosynthesis (**Figure 1**). While the non-differentiated Thp-1 cells did not produce detectable amounts of IL-1β in response to *H. pylori* at early time points below 8 h (not shown), they released low amounts of IL-1β at 20 h co-incubation, which was significantly higher than under the mock-co-incubated conditions (**Figure 1**). IL-1β release was less associated with the CagT4SS, and *cag*PAI and heptose (*hldE*) mutants still provoked a significantly higher IL-1β release by Thp-1 cells than mock conditions. In the setting without prior cell priming, the differential proinflammatory response of phagocyte-like cells to *H. pylori* was exquisitely MOI-dependent (**Supplementary Figure S2**), although the heptose mutants activated the cells significantly less than wild type bacteria at all MOIs up to 25. Various wild type strains at the same MOI activated the Thp-1 cells to a significantly different extent at 20 h post co-incubation (p.c.) (**Figures 1C–E**). *cagA* mutants did not show a significant difference in cell activation in comparison to wild type bacteria in any of the settings, while Δ*cag*PAI mutants or T4SS-inactive (*cagY*) mutants showed a significantly reduced activation potential (IL-8 release) as

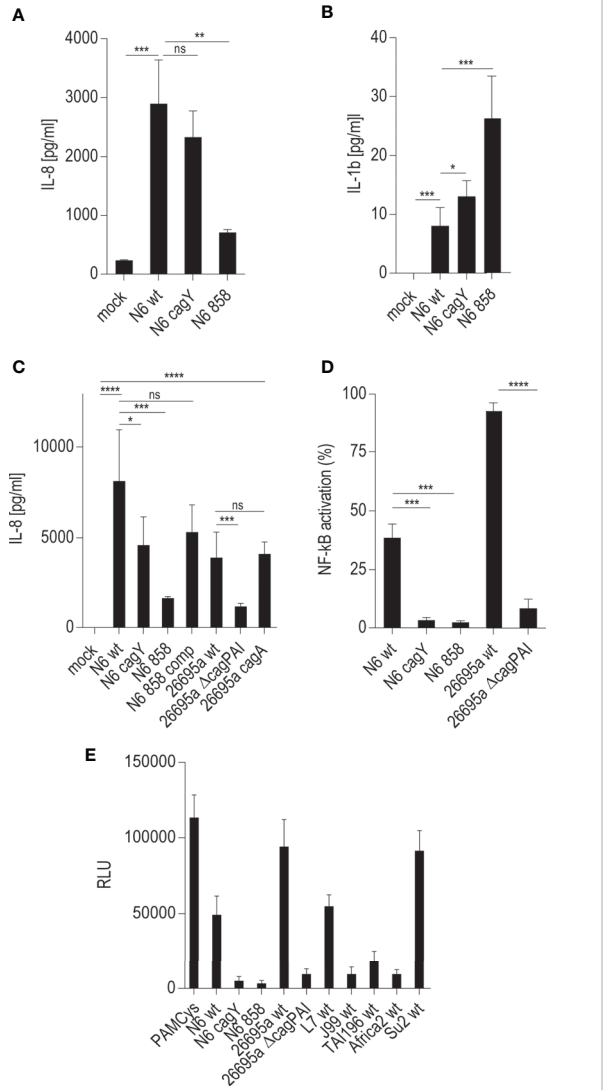

FIGURE 1 | Significant role of LPS inner core heptose biosynthesis and the *cag*PAI in co-incubated Thp-1 cells activated by *H. pylori* or its isogenic mutants. In **(A)**, the IL-8 secretion by Thp-1 cells co-cultured with strain N6 wild type (N6 wt) and isogenic *cagY* and *hldE* (HP0858 – 858) mutants as a marker for cell activation is shown (20 h co-incubation, MOI = 5, no pre-activation). **(B)** low IL-1β secretion in Thp-1 cells co-incubated with the same set of strains at MOI = 10, 20 h (no IL-1β was detected at MOI 5). **(C)** comparison of IL-8 secretion in Thp-1 cells co-incubated with two different *H. pylori* strains (N6 and 26695a) and their isogenic mutants (MOI = 25, 21 h). **(D)** Isolated quantitation of NF-κB activation using Thp1_luc cells, containing firefly luciferase gene under the control of a high-affinity NF-κB promoter (Methods). Thp1_luc cells were co-incubated for 4 h with N6 and 26695a strains and their respective mutants (MOI = 10) as indicated. The values are shown as relative luminescence values, normalized to the activation by heptose-independent, reference substance PAMCys (TLR2 agonist; 20 ng/50 µl), which was set to 100%. **(E)** Quantitation of Thp1_luc NF-κB activation by various *H. pylori* strains (MOI = 10) shown as arbitrary luminescence units (RLU). Comparative values of activation by the PAMCys reference are indicated. One representative independent biological experiment out of three is depicted in each panel. Statistical significance of differences was calculated using unpaired student's *t*-test. Significant p values: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns is non significant.

compared to the parental wild type strain (**Figure 1**). It is known that bacterial heptose metabolites such as ADP-heptose can activate cellular NF-κB at early time points (16, 17, 21, 59), which, in gastric epithelial cells co-incubated with live *H. pylori*, is mediated by an active CagT4SS (17). Subsequent results that we gathered from co-incubation experiments with a Thp1_luc NF-κB luciferase reporter cell line, which allows to specifically quantitate NF-κB activation (54), underlined that, also in the monocyte/macrophage cells, the early NF-κB response induced by live *H. pylori* is strongly heptose- and CagT4SS-dependent; heptose biosynthesis mutants (*hldE*), Δ*cag*PAI (complete T4SS-deficient) and *cagY* (T4SS functionally deficient) mutants were strongly impaired in inducing NF-κB activation (**Figure 1D**). In addition, we determined a remarkable diversity between different *H. pylori* wild type strains to induce NF-κB specifically in the non-preprimed luciferase reporter cells (**Figure 1**). Live *H. pylori* also activated NF-κB in a strongly CagT4SS- and heptose-dependent manner (**Figure 1**). This effect paralleled the cytokine measurements, but clearly showed a more distinct difference between wild type and the *cag*PAI (*cagY*) or *hldE* mutant (**Figure 1**). *hldE*-complemented bacteria recovered the cell activation properties on Thp-1 cells (**Figure 1E**). In an alternative set-up, we also primed the Thp-1 cells prior to bacterial co-incubation using PMA (47, 53) or *Escherichia coli* LPS (not shown). While co-incubation with live *H. pylori* activated the pre-primed cells to produce increased amounts of IL-8 and more IL-1β as compared to mock-coincubation (**Supplementary Figures S1A, B**), both *H. pylori* wild type and all different mutants strongly and uniformly activated the cells. No significant reduction in cytokine release was observed for the CagT4SS mutants or the heptose biosynthesis mutants as compared to wild type bacteria in this setting (**Supplementary Figures S1A, B**). Interestingly, in the primed setting, for IL-1β release, the ADP-heptose-negative *hldE* mutant (HP0858-mut) showed even a significant increase in IL-1β release over the wild type bacteria, similar but stronger than in the non-primed conditions. However, in general IL-1β release in these settings was rather low, with or without PMA activation.

We also addressed the question, whether co-incubation with live *H. pylori* differentiated the Thp-1 monocytes rather to an M1-like (proinflammatory) or M2-like macrophage phenotype (44, 60–62). M1 macrophages tend to produce higher amounts of IL-6 (and IL-8) while M2 macrophages predominantly produce IL-10, less IL-6 or IL-8 (63). M1 tend to upregulate STAT1, STAT2, and IRF5, while M2 rather increase STAT3, STAT6, IRF3, and IRF4 (62). Thp-1 co-incubated with *H. pylori* (at MOI 10) produced copious amounts of IL-8 and, to a lesser extent IL-6 (**Supplementary Figure S3**), but produced no (at 8 h p.c.) or very low (at 20 h p.c.) amounts of IL-10. A trend to differentiation towards a proinflammatory M1 macrophage phenotype was also supported by the transcriptome results (see below), which showed an increased expression of M1-related upstream activator and M1-specific transcription factor genes such as STAT1, STAT2, and AP-1 (64) as well as IRF5 (62), while M2-specific STAT6, IRF3, and IRF4 transcripts were rather downmodulated or not increased (**Supplementary Figures S4A, B**).

Early Activation of Cellular Signaling by *H.pylori* in Thp-1 Cells Is Directly Modulated by Heptose Metabolites and an Active CagT4SS; Free Heptose and Bacterial ETLs Can Activate in the Absence of an Active T4SS

Since live *H. pylori* bacteria activated monocytes to a macrophage-like phenotype, in a partially *cag*PAI- and heptose-dependent manner, we sought to determine whether the presence of the Cag injection apparatus or an injected molecule, most likely ADP-heptose, was the main contributing factor. We prepared *H. pylori* bacterial supernatants and bacterial enzyme-treated lysates (ETL) (17), enriched in various metabolites including heptoses (21), and comparatively tested pure ADP-heptose. Pure ADP-heptose dose-dependently activated Thp-1 cells to produce high amounts of IL-8 (**Figure 2**), but not IL-1β or IL-10 (not shown), at 20 h p.c. Since cellular NF-κB activation was directly correlated to prior ADP-heptose activation in previous studies of epithelial cells (16, 17, 21), we complemented this approach using the Thp1_luc NF-κB-luciferase reporter cells, which provide a direct and high-throughput detection and quantitation system for NF-κB activation. Pure ADP-heptose directly activated NF-κB in the Thp-1 reporter cells to a high extent at 4 h post-co-incubation and at later time points (not shown), similarly to PAMCys (TLR2 ligand) which we used as normalization and positive control condition (**Supplementary Figure S3A**).

In human epithelial cells, the CagT4SS seems to be required to transport heptose metabolite(s) into the cells when exposed to live bacteria (17). However, free heptoses can activate epithelial cells in the absence of bacteria (21). It was not known before for monocyte/macrophage-like cells, whether bacterial supernatants or bacterial metabolite-enriched lysates are able to activate in the absence of live bacteria. In order to determine, whether the soluble metabolites, including LPS heptose metabolites, produced by *H. pylori* are able to activate Thp-1 cells independently of transfection or a transport system, we prepared bacterial supernatants and ETL lysates (17), from various *H. pylori* strains and mutants. We co-incubated Thp-1 NF-κB luciferase reporter cells with pure ADP-heptose, with selected *H. pylori* ETLs from wild type and mutants, or with bacterial supernatants. Pure, externally added ADP-heptose activated the cells and NF-κB signaling to a high extent and concentration dependently at 4 h post-co-incubation (**Figure 2**, **Supplementary Figure S3A**). Similarly, ETLs from wild type *H. pylori* added to the cell culture medium strongly activated NF-κB (**Figures 2B**). This activation potential in Thp-1 cells was very similar for ETLs derived from wild type strains and CagT4SS-deficient mutants (**Figure 2C**). In contrast, ETLs generated from heptose-negative *hldE* mutants activated NF-κB to a significantly lower extent (**Figure 2**), only slightly more than the activation recorded in mock-co-incubated cells. Bacterial supernatants, heptose-dependently and strain-dependently, also activated Thp-1 luciferase cells, but considerably less than ETLs (**Supplementary Figure S3C**), indicating that the intact bacteria grown in the absence of cells release only low amounts of free heptose metabolites into the medium. Free NOD1 or NOD2 ligands as well as purified bacterial DNA (TLR9 ligand) did not activate the Thp-1 cells in this setting (**Supplementary Figure S4**), indicating that NOD and TLR9 signaling do not play a role to activate Thp-1 cells.

In order to block phagocytic uptake, the cytoskeleton modulator cytochalasin D (CytD) was used in co-incubation experiments. CytD inhibited the induction of IL-8 secretion in Thp-1 cells co-incubated with pure ADP-heptose, however only by about 30% (**Supplementary Figure S3D**). This was also true for PAMCys co-incubation, exemplarily used with the intent to activate the IL-1/TLR (TLR2) response pathway (**Supplementary Figure S3D**). Similarly, *cag*PAI- and heptose-dependent effects of NF-κB induction upon co-incubation with *H. pylori* ETLs or live *H. pylori* in Thp-1 cells were partially, but not completely, inhibited by CytD. We also addressed the question, whether phagocytic uptake in general was influenced by heptose metabolite exposure of the cells. We incubated Thp-1 cells with microbeads in the absence or presence of pure ADP-heptose for 20 h (**Supplementary Figure S4E**). Beads were taken up by the cells and no significant difference in bead uptake between heptose-co-incubated or heptose-free beads was determined (**Supplementary Figure S4E**).

Primary Human Monocytes and Matured Primary Macrophages Are Activated by Pure ADP-Heptose, and *H. pylori* Heptose Biosynthesis Competence Codetermines Activation Potential

In order to verify that *H. pylori* can activate primary human phagocytes in a heptose-dependent manner, as determined before in co-incubated Thp-1 cells for pure ADP-heptose and bacteria, we isolated primary CD14-positive monocytic cells from human peripheral blood. We co-incubated the purified primary human monocytes directly with pure ADP-heptose, without further maturation, at different concentrations for 20 h (**Supplementary Figure S5**). The outcome clearly demonstrated a significant and concentration-dependent activation of the non-differentiated CD14+ cells by pure ADP-heptose (**Supplementary Figure S5A**), similar to the co-incubated Thp-1 cells. In addition, we differentiated the CD14+ cells to human monocyte-derived macrophages (hMDMs) for seven days by pre-incubation with M-CSF, and then subjected them to pure ADP-heptose at different concentrations (**Supplementary Figure S5B**), or co-cultured them with live bacteria, for 4 h. In the matured hMDMs, we observed as well a significant activating effect of pure ADP-heptose (**Figure 2D**, **Supplementary Figure 5B**). hMDMs co-incubated with live bacterial strains or treated bacterial lysates enriched in metabolites (ETL) showed a clear difference between a heptose-positive wild type strain and an isogenic *hldE*- mutant (**Figure 2D**, **Supplementary Figure 5C**). These activation effects were similar for primary monocytes and differentiated hMDMs obtained from independent donors.

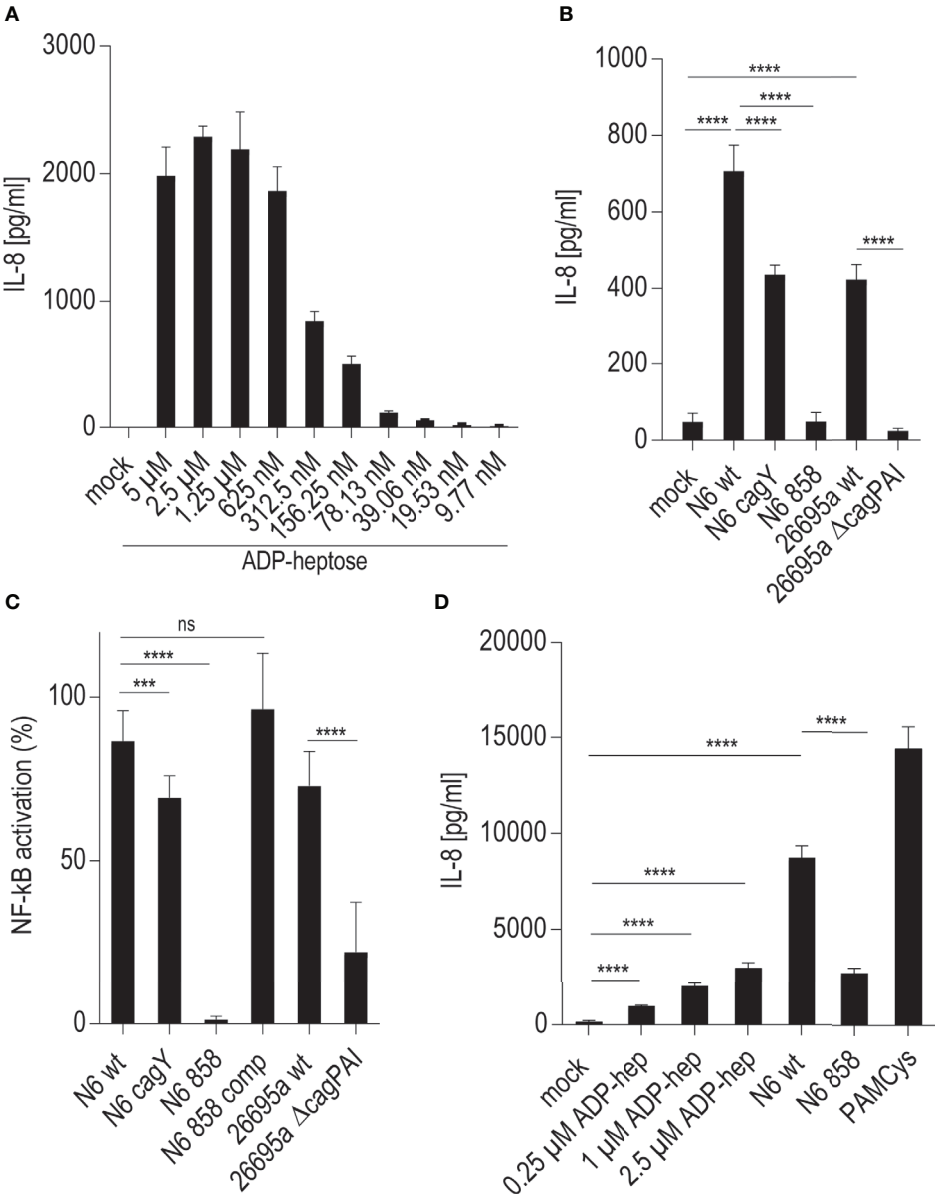

FIGURE 2 | Activation of monocyte/macrophage cell line Thp-1 and primary human macrophages by pure ADP-heptose, enzyme-treated bacterial lysates (ETL), and live *H. pylori*. **(A)** dose-dependent activation of Thp-1 cells by pure ADP-heptose: ADP-heptose in different concentrations as indicated was applied to Thp-1 monocyte/macrophages for 20 h in 24-well plates, and IL-8 secretion was quantitated using ELISA (for comparison, concentration-dependent activation of NF-κB reporter cells Thp1_luc by ADP-heptose is shown in **Supplementary Figure S3A**). **(B)** Thp-1 cells were co-incubated with ETLs of two *H. pylori* strains and their isogenic *cagY*, Δ*cag*PAI, and *hldE* mutants for 20 h in 24-well plates (50 µl of lysates per well added to cells), followed by the quantitation of IL-8 secreted in the cell supernatant by ELISA. **(C)** ETLs prepared from two different *H. pylori* strains including their isogenic heptose mutant (N6 858) and complementant (N6 858 comp), *cagY* and *cag*PAI-deletion mutant ETLs were comparatively tested for NF-κB induction in 96-well format (Thp1_luc cells, 4 h co-incubation, 2.5 µl of lysate/well). Relative values in %, normalized to an independent reference (PAMCys) which was set to 100%, are depicted. *cagY* ETL led only to a mild reduction of NF-κB compared to wt ETL, while *cag*PAI mutant ETL was associated with a strong reduction of activation. ETLs or pure ADP-heptose alone did not induce significantly higher IL-1β secretion than mock-co-incubated cells under the tested conditions, while PAMCys induced IL-1β (not shown). No pre-activation or priming was used in these assays. One of at least three independent biological experiments is shown in each panel. **(D)** Primary human monocyte-derived macrophages (hMDMs) were treated with pure ADP-heptose in different concentrations (hep; as indicated), with PAMCys (400 ng/ml) and with live bacteria (MOI 5; N6 wt is *H. pylori* N6; N6 858 is isogenic *H. pylori hldE* mutant deficient in LPS core heptose biosynthesis) in 24-well plates. Cell activation was quantitated by IL-8 ELISA. Primary macrophages were significantly activated by ADP-heptose in a concentration-dependent manner. Biosynthesis-deficient *hldE* mutant activated significantly less than *H. pylori* wild type bacteria. Mean and standard error of six replicates is shown for each condition. Statistical significance of differences in **(A–D)** was calculated using unpaired student's *t*-test. Significant p values: ****$p < 0.0001$; ***$p < 0.001$; ns is non significant.

Regulation of the Genome-Wide Transcriptome in Thp-1 Cells by *H. pylori* Is Strongly Dependent on the LPS Core Heptose Pathway and Is Partially Recapitulated by Pure ADP-Heptose

In order to obtain a comprehensive overview and compare transcript regulation induced by free ADP-heptose or by live *H. pylori* and its heptose metabolites in Thp-1 cells, we harnessed global transcriptome assays. Initially, we assessed the activity of free ADP-heptose co-incubation on the complete Thp-1 cell transcriptome. For this purpose, non-pre-differentiated Thp-1 cells were treated with free ADP-heptose (2.5 μM) for 8 h, RNA was isolated, and the comprehensive cellular transcriptome was analyzed by deep sequencing (RNA-Seq, Methods). For comparison to this primary core dataset, live *H. pylori* wild type bacteria and isogenic heptose biosynthesis mutants (*hldE*) were also co-incubated with the non-primed Thp-1 cells for 8 h. cDNAs generated from all these settings were then also subjected to transcriptome sequencing and analyzed. In this comprehensive analysis, ADP-heptose-co-incubated cells showed numerous differentially regulated transcripts in comparison to mock-co-incubated, which was directly related to ADP-heptose activation (**Figures 3A, M, N**; **Supplementary Table S1**). These experiments confirmed again that cell transfection was not necessary to provide ADP-heptose access to those cells. Signature transcripts of Thp-1 enhanced by ADP-heptose or live bacteria at the early time points encompassed il-8 (cxcl8), ccl2, ccl3, ccl4, and il-1β, but not il-6 or il-10 (**Figure 3D**, **Table 3** and **Supplementary Tables S1, S2**). We also detected a substantial overlap of transcripts regulated by co-incubation of Thp-1 cells with either pure ADP-heptose or with live bacteria *of H. pylori* wild type (**Figure 3B**, **Table 4** and **Supplementary Table S2**). Dominantly regulated transcripts by cell co-incubation with pure ADP-heptose (cut-off of eight-fold regulated) included a strong upregulation of NF-κB subunit genes for NFKB2 and RELB and downstream IL-8 and complement factor C3. Likewise, transcriptional coactivator BCL3, and OAS2, OAS3 genes involved in innate recognition of double-stranded RNA (65), as well as CD40 and a macrophage glycan transporter gene of unknown specificity, SLC2A6, were strongly upregulated (**Figure 3**, **Table 4**). While about 340 differentially regulated transcripts (**Figure 3M**, **Supplementary Table S2**) overlapped between ADP-heptose-co-incubated cells and *H. pylori* wild type bacteria-exposed cells at a cut-off of two-fold regulated, pure ADP-heptose treatment (ca. 852 transcripts; **Tables 1, 2**) and Cag-positive *H. pylori* wild type bacteria (419 transcripts, **Supplementary Table S2**) also regulated a subset of specific transcripts differentially. Overlapping and distinct transcript regulation was also identified for a comparison of the respective comprehensive transcriptomes, between the genes differentially regulated over mock conditions by the *hldE* mutant and the parental wild type bacteria (**Supplementary Figure S6**, **Supplementary Table S2**). Numerous genes were differentially regulated between *H. pylori* wild type-co-incubated and *H. pylori* heptose-mutant-co-incubated cells (**Figure 3**). As expected, il-8 was among the differentially regulated genes/transcripts between mock and wild type bacteria as well as between ADP-heptose-co-cultured and mock cells, and between *hldE* and wild type bacteria-exposed. Despite the clear differences in transcript regulation between *H. pylori* wild type and *hldE*-mutant co-incubated cells, we also found a substantial overlap of regulation between those two conditions over mock, which were not quite comparable in strength of regulation, but encompassed a high number of similarly up-or downregulated transcripts (**Supplementary Figure S6**; **Supplementary Table S2**). Among the most strikingly differentially regulated transcripts between *H. pylori*-exposed and free heptose-treated cells was the downregulation of PYCARD/ASC transcript for live *H. pylori versus* mock, and the upregulation of the same transcript for the ADP-heptose-treated cells *versus* mock-treated.

In order to compare transcript regulation for a wider range of cell-co-incubated mutants, a selected panel of transcripts, mainly such involved in the innate immune response, collected from the comparison of global transcriptomes, were subsequently verified using cDNAs generated from Thp-1 cells co-incubated with various *H. pylori* mutants (live bacteria) (**Figures 3C–L**). Amounts of transcripts of downstream regulated cytokines IL-8, IL-1β; and CCL4 were comparatively determined by qPCR for the *cagY* mutant (HP0527; CagT4SS functionally deficient), and the heptose HP0858 (*hldE*) mutant in addition to cells co-incubated with the parental bacteria (**Figures 3C–E**). We determined significant differences between the conditions, in particular between wild type- and mock- or HP0858 (*hldE*) mutant-co-incubated cells (decrease of cytokine transcripts). Interestingly, in qPCRs, which test for transcript amounts of cytokine genes (il-1β and il-8) as a marker for transcription factor, e.g. NF-κB, activation, not for cytokine release, the *cagY* mutant presenting with a defective CagT4SS did not show reduced IL-8 or IL-1β transcript amounts in comparison to wild type, in contrast to cytokine release. In contrast, the ADP-heptose-deficient HP0858 mutant always displayed reduced transcript amounts for those specific cytokine transcripts. This data strongly indicates that for cytokine transcript induction in the presence of free heptose metabolites, no active T4SS is required (indicating active metabolite uptake), whereas for cytokine release by live bacteria (as shown by ELISA in **Figures 1, 2**), even when the bacteria actively produce heptoses, an active bacterial transport system, provided by the CagT4SS, is necessary to strongly promote cytokine release. Other activities by the CagT4SS may influence cell/caspase-1 activation for cytokine release. Deficiencies in cellular cytokine release induced by the heptose HP858(*hldE*) mutant bacteria in contrast to the wild type bacteria, as outlined in the previous paragraphs, are therefore likely to result rather from a reduction of induction of primary cytokine transcript than from a defect of the CagT4SS. Defects of the *H. pylori hldE* mutant in cytokine transcript upregulation were completely reverted by complementing the HP0858 gene *in trans* in another (*rdxA*) locus of the bacterial chromosome (17). Transcription factor NF-κB subunit transcript (NFKB2) was very strongly induced by both pure ADP-heptose and wild type *H. pylori*, but not by the *hldE* mutant in these settings, while TNFα transcript was not

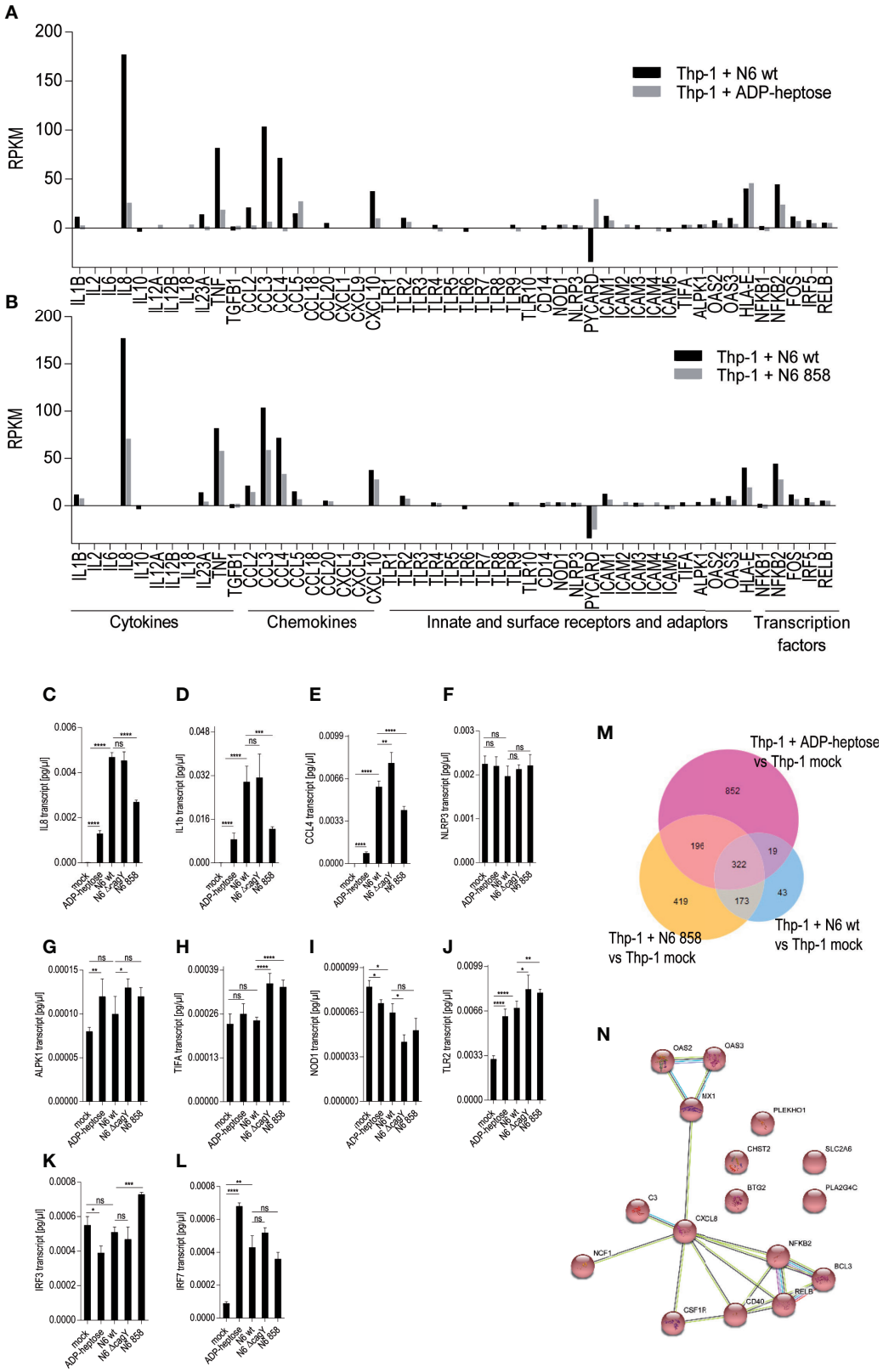

FIGURE 3 | Continued

FIGURE 3 | Transcriptional activation of monocyte/macrophage cell Thp-1 by live *H. pylori* and pure ADP-heptose. We performed comprehensive transcriptome assays (RNA-Seq) of cells that were co-incubated for 8 h with *H. pylori* strains and pure ADP-heptose (selected results in **Table 4**, **Supplementary Tables S1, S2**, and full results under project No. PRJNA685657 deposited at ncbi). **(A)** Selected results (panel of innate immune-related genes) of differential transcript amounts (RPKM relative to mock-co-incubated conditions; mock values were subtracted) after 8 h of co-incubation of *H. pylori* N6 wt live bacteria (MOI = 10, black bars) and ADP-heptose (5 µM; grey bars). **(B)** Selected results (panel of innate immune-related genes) of differential transcript amounts (RPKM relative to mock-co-incubated, mock values were subtracted) after 8 h of Thp-1 co-incubation of *H. pylori* N6 wt live bacteria (MOI = 10, black bars) and *hldE* mutant bacteria (MOI = 10; grey bars). Panels **(C–L)** show qPCR results of selected genes by Thp-1 cells co-cultured with live N6 wild type and mutants, also including the *cagY* mutant, which was not included in the RNA-seq analysis. **(C–E)** show qPCR results for downstream activated cytokine genes il8, il1b, and ccl4; **(F–L)** show genes coding for relevant pattern recognition receptors, adaptors, and transcription factors: ALPK1, TIFA, NLRP3, TLR2, NOD1, IRF3, IRF7. Results are quantitated as absolute values [pg/µl], normalized to human GAPDH transcript for each condition. Statistical differences were calculated by unpaired student's *t*-test. **(M)** Venn diagram comparing the differentially expressed genes between live *H. pylori* N6 wt bacteria *versus* mock-co-incubated (blue circle), live *H. pylori hldE* (HP0858) mutant-co-incubated *versus* mock (orange), as well as ADP-heptose-treated cells *versus* mock (pink circle). The genes contained in the intersection of comparisons (overlap of differentially expressed genes) between wt bacteria-activated and ADP-heptose- activated cells are shown in **Supplementary Table S2**). **(N)** Intersection of Venn diagram of two RNA-Seq experiments of ADP-heptose-co-incubated Thp-1 cells vs. mock-co-incubated, visualized as a pathway map in STRING (all are upregulated transcripts, colored in red). The threshold was set to 8-fold change to visualize a reduced selection of regulated genes. The table view for this comparison is shown in **Table 4** (for all genes regulated above a four-fold cut-off) and in **Supplementary Table S2**. All other comparisons were performed with a threshold of (+/-)2-fold regulated. (For selected extended results see Excel **Supplementary Table S1**). Significant p values for differences shown in **(C–L)** were calculated by unpaired Student's *t*-test: ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns is non significant.

induced by ADP-heptose and not differently induced between parental wild type bacteria and *hldE*- bacteria. Comparative qPCR for a selection of genes coding for upstream receptors involved in heptose sensing and other innate immune pathways activated by *H. pylori* bacteria (NLRP3, ALPK1, TIFA, NOD1, TLR2; **Figures 3F–J** and **4**) revealed that transcripts for these innate immune pattern recognition receptors (PRR) were not substantially changed under any of the tested co-incubation conditions over mock.

Except for TLR2, which was always strongly active in Thp-1 cells (see PAMCys controls used in most assays), the above-mentioned PRR as well as NOD2, TLR4, and TLR9 were not highly activated in Thp-1 cells (**Supplementary Figures S4C, D**). Mining the transcriptomes for M1- and M2-macrophage-specific signatures of transcripts in the transcriptomes revealed that co-culture of the Thp-1 cells with either wild type *H. pylori* or free ADP-heptose seemed to emphasize the induction of M1-related transcripts (**Supplementary Figures S4A, B**). IFN-γ was not produced in the supernatants of heptose- or bacteria-co-incubated Thp-1 cells (data not shown), which suggested that JAK/STAT pathway or IRF transcription factors were not activated under the short-term co-incubation conditions used. Finally, the question needed to be answered whether the TIFA-ALPK1 pathway (21) is responsible for heptose-mediated activation in monocyte/macrophages. Using siRNA knock-down of TIFA, the activity of pure ADP-heptose on the cells was significantly reduced to more than half of the siRNA negative control (determined by luciferase reporter and IL-8 secretion; **Figures 5A, B**). At the same time, we were able to reduce TIFA transcript in difficult-to-transfect Thp-1 cells by about 30% (**Figure 5C**). These assays confirmed TIFA as an important mediator of responses against ADP-heptose in Thp-1 monocytic cells.

Heptose-Dependent Activation of Cellular Signal Transduction by *H. pylori* in Monocyte/Macrophage-Like Cells Is Not Influenced by NLRP3

We next investigated whether one major intracellular innate immune sensor, NLRP3, a central inflammasome activator which has been characterized to be strongly activated in macrophages by *H. pylori* (14, 15), contributes to or interferes with the early heptose-dependent signaling and cytokine release observed in professional phagocytes, as we reported above. This approach was also designed to test whether heptose metabolites are involved in the NLRP3-dependent response, and whether NLRP3 can feed back into or synergize directly with the (ADP-)heptose-dependent signaling pathway. For this purpose, we utilized a NLRP3-deficient (NLRP3-def) Thp1 cell line in comparison to the Thp-1 wild type macrophage line. When we co-cultured the NLRP3-def cells with *H. pylori*, we observed, as expected, only a very low response of inflammasome-dependent IL-1β (cytokine release and transcript response; **Figure 4**), to live bacteria co-incubation at different time points, in comparison to the wild type, NLRP3-competent, cells. The heptose-deficient and *cag*PAI mutants did not show a significant difference in IL-1β release on the NLRP3-def cells compared to the parental wild type bacteria. In the quantitative IL-8 assays, overall cytokine release was even considerably stronger in the NLRP3-deficient cells than in the NLRP3-competent Thp-1 parent, for co-incubation with live bacteria, or in parallel experiments with ETLs of the same strains. This result indicated no activating role of NLRP3 in promoting the IL-8 output triggered by *H. pylori* in this cell type. Interestingly, the *H. pylori* HP858/*hldE* mutant also activated the NLRP3-deficient cells to produce IL-8, albeit lower than for wild type bacteria, but very likely higher than in Thp-1 parental cells. Even if a direct comparison between two different separately co-incubated cell lines is not precise, we determined that pure ADP-heptose showed roughly the same absolute activation levels in NLRP3-deficient Thp-1 cells as in parental Thp-1. These results suggest that, while TIFA, as a response adaptor to heptose metabolites, can increase NLRP3 responses as reported in previous studies (66), lack of NLRP3 has a limited influence on ALPK1-TIFA signaling by ADP-heptose or by *H. pylori*, in particular on the outcome of IL-8 release and NF-κB activation in these macrophage-like cells. The LPS heptose recognition pathway by ALPK1-TIFA seems therefore not to be positively or negatively influenced by NLRP3.

TABLE 4 | Selected human gene transcripts regulated by ADP-heptose co-incubation of human monocyte/macrophage cells line Thp-1 (cut-off of fourfold regulated in comparison to mock, from two independent experiments [biological replicates]).

Name	Thp-1 ADP-heptose *vs.* Thp-1 mock (A2B2)					Thp-1 + ADP-heptose *vs.* Thp-1 mock (ZA1)				
	Max group mean (RPKM)	Fold change	P-value	FDR p-value	Bonferroni	Max group mean (RPKM)	Fold change	P-value	FDR p-value	Bonferroni
SLC2A6	6.59	92.71	2.13E-06	5.50E-04	0.08	10.85	43.8	6.81E-14	1.01E-11	2.50E-09
OAS3	1.06	39.57	1.82E-07	6.59E-05	6.66E-03	4.1	30.75	0	0	0
PLA2G4C	0.29	31.58	3.48E-04	0.04	1	0.8	25.26	4.10E-10	3.55E-08	1.50E-05
BCL3	3.09	22.37	1.42E-07	5.62E-05	5.22E-03	7.85	26.25	1.11E-16	2.31E-14	4.07E-12
IL8	11.03	22.05	1.64E-07	6.14E-05	6.02E-03	26.84	24.76	2.22E-16	4.50E-14	8.15E-12
CHST2	6.09	20.53	2.24E-05	4.08E-03	0.82	7.19	48.25	5.44E-05	1.46E-03	1
RELB	2.8	18.2	0	0	0	5.11	19.32	0	0	0
C3	0.69	14.01	4.65E-07	1.47E-04	0.02	3.83	17.78	0	0	0
CSF1R	0.45	12.78	1.23E-06	3.40E-04	0.05	2.17	12.2	0	0	0
MX1	1.62	11.1	9.13E-14	1.04E-10	3.35E-09	9.25	38.77	0	0	0
OAS2	0.67	10.57	8.30E-06	1.73E-03	0.3	4.95	21.15	0	0	0
CD40	2.94	9.88	4.67E-10	2.95E-07	1.71E-05	6.06	27.45	0	0	0
NFKB2	19.06	9.26	0	0	0	25.58	14.96	0	0	0
C1orf147	2.1	8.78	3.63E-04	0.04	1	6.21	4.45	1.11E-07	6.28E-06	4.09E-03
NCF1	4.56	8.54	6.22E-15	8.45E-12	2.28E-10	17.92	33.25	0	0	0
BTG2	7.22	8.11	4.57E-07	1.46E-04	0.02	24.38	10.19	0	0	0
PLEKHO1	6.75	8.1	6.22E-15	8.45E-12	2.28E-10	6.53	12.71	2.89E-15	5.02E-13	1.06E-10
FEZ1	1.22	7.83	7.77E-16	1.19E-12	2.85E-11	2.32	5.05	0	0	0
GAS7	0.34	7.73	0	0	0	0.74	7.86	0	0	0
FTHL16	367.92	7.43	0	0	0	997.03	13.9	0	0	0
NCF1B	2.22	7.33	9.22E-08	3.89E-05	3.38E-03	4.6	13.09	1.22E-15	2.27E-13	4.48E-11
RP11-274P12.1	596.02	6.94	0	0	0	1,535.62	17.35	0	0	0
RP4-646B12.2	844.59	6.87	0	0	0	1,637.92	11.85	0	0	0
TNFAIP3	7.66	6.8	0	0	0	13.81	10.28	0	0	0
IFI6	4.43	6.61	1.76E-06	4.59E-04	0.06	38.61	34.82	0	0	0
PARP14	1.08	6.21	2.01E-11	1.64E-08	7.36E-07	5.52	12.39	0	0	0
NFKBIA	47.84	5.94	0	0	0	71.83	10.19	0	0	0
PDGFA	1.59	5.93	8.94E-08	3.81E-05	3.28E-03	0.98	6.58	7.73E-06	2.67E-04	0.28
CYBB	6.78	5.7	0	0	0	5.19	7.62	0	0	0
LIMD2	35.69	4.98	0	0	0	66.45	6.31	0	0	0
TRIM16L	1.61	4.89	3.39E-05	5.67E-03	1	3.62	9.46	3.00E-11	3.08E-09	1.10E-06
AKR1C1	4.08	4.76	2.42E-11	1.85E-08	8.87E-07	6.72	5.74	0	0	0
MSC	8.89	4.65	1.26E-05	2.50E-03	0.46	24.67	24.64	2.41E-14	3.83E-12	8.84E-10
CD44	0.35	4.59	1.57E-06	4.17E-04	0.06	0.57	8.34	2.75E-12	3.29E-10	1.01E-07
CD83	1.76	4.42	1.52E-06	4.11E-04	0.06	2.08	4.58	1.15E-07	6.43E-06	4.21E-03
LPXN	0.62	4.23	6.27E-06	1.39E-03	0.23	2.93	18.45	0	0	0
MT2A	68.46	4.21	7.99E-11	5.53E-08	2.93E-06	172.54	9.53	0	0	0
TFEB	0.65	4.15	2.56E-06	6.49E-04	0.09	1.37	6.73	3.44E-15	5.93E-13	1.26E-10
TYMP	32.11	4.15	0	0	0	63.05	12.66	0	0	0
ME1	0.53	4.13	0	0	0	1.28	4.62	0	0	0
OLIG2	17.97	4.1	3.40E-10	2.23E-07	1.25E-05	48.07	5.41	0	0	0
XAGE1B	6.71	-6.55	1.48E-07	5.79E-05	5.44E-03	5.9	-4.59	6.21E-06	2.19E-04	0.23
RP11-350E12.1	5.82	-9.53	2.00E-04	0.03	1	18.39	-28.66	1.11E-08	7.51E-07	4.05E-04

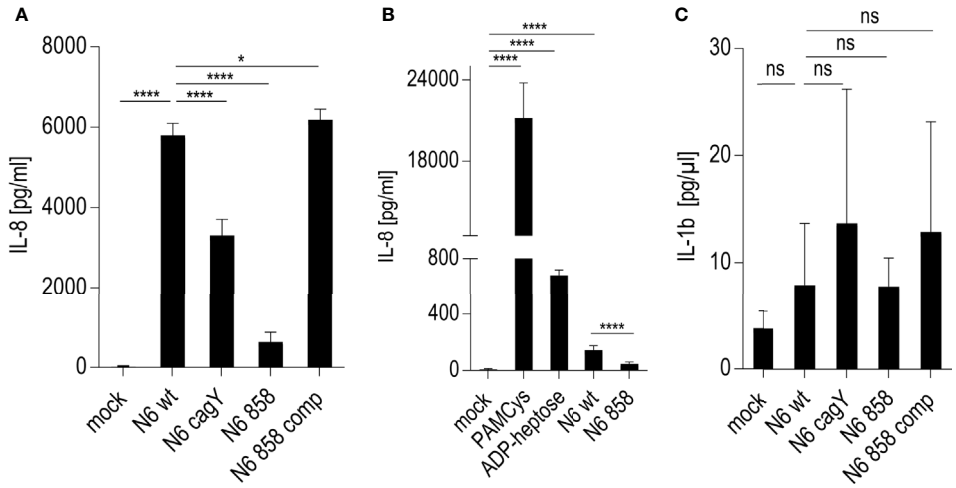

FIGURE 4 | Response of NLRP3-deficient Thp-1 monocyte/macrophage cells (Thp-1 dNLRP3) to live *H. pylori* and pure ADP-heptose. **(A, B)** show IL-8 secretion by co-incubated, NLRP3-deficient Thp-1 cells. **(A)** Thp-1 NLRP3def cells co-incubated with live *H. pylori* bacteria of different genotypes at MOI 5 for 20 h in 24 well format **(B)** Thp-1 dNLRP3 cells co-incubated with *H. pylori* ETL (50 µl/well) from OD_{600} = 2 in 24-well plate for 20 h. As controls, for TLR (3) activation, PAMCys at 400 ng/well, and for ALPK1 activation, ADP-heptose at 5 µM, respectively, were added in parallel experimental conditions. **(C)** IL-1β secretion by NLRP3def Thp-1 cells co-incubated with live *H. pylori* variants as in **(A)**. Results of one representative experiment out of three independent experiments are shown in each panel. Cell responses in **(A–C)** were quantitated using cytokine ELISA. Cells were not primed before adding the respective stimuli. IL-8 was not decreased (rather increased) in NLRP3-deficient cells upon *H. pylori* co-incubation, while IL-1β was significantly decreased and lost the CagT4SS-and heptose-dependent phenotype upon deficiency of NLRP3. Statistical differences were calculated by unpaired student's *t*-test. Significant p values: ****$p < 0.0001$; *$p < 0.05$; ns is non-significant.

DISCUSSION

The aim of this study was to investigate the role of LPS heptose-mediated signaling by *H. pylori* in human monocyte-like cells, which can be differentiated to a macrophage-like phenotype, exemplified by the model cell line Thp-1 (47, 53, 67). The recently discovered heptose-dependent innate recognition and signaling process (68) had not been studied specifically in human phagocytic cells. We and others have recently demonstrated that human epithelial cells are selectively activated at early time points by Gram-negative bacteria through heptose metabolites of the LPS inner core biosynthesis, which mediate intracellular activation *via* the ALPK1-TIFA axis (17, 18, 59, 68, 69).

In epithelial cells co-incubated with *H. pylori*, this TIFA- and ALPK1-mediated signaling process is CagT4SS-dependent and LPS heptose metabolite-dependent (16, 17). More recently, further biochemical analyses have refined that the bacterial LPS heptose metabolite that is predominantly and directly recognized by the cellular kinase ALPK1 seems to be predominantly the activated inner core LPS metabolite ADP-heptose (21, 22, 70). ADP-heptose, in *H. pylori* and other Gram-negative bacteria, is specifically generated by the second biosynthesis step catalyzed by the bifunctional enzyme RfaE/HldE (HP0858 in *H. pylori* strain 26695) (17, 21). For *H. pylori*, ADP-heptose or potentially other heptose metabolites are transported into human epithelial cells by the CagT4SS (17). So far, it has been established that monocytic cells or activated macrophages isolated from mice seem to recognize *H. pylori* mainly *via* TLR2, TLR4, TLR9, NOD2, and NLRP3 activation (11, 14, 15). In addition, previous published work has also found a contribution of the CagT4SS in the early activation (TLR independent) of macrophage-like cells, exemplified by results collected for Myd88-Trif-deficient primary bone-marrow-derived mouse macrophages (44). In this prior work, the authors described that anergic, broadly TLR signaling-deficient mutant macrophages recognized the *H. pylori* CagT4SS by innate immune activation and that this was a rather NOD1/NOD2- and Rip2-independent process, although the authors could not attribute it to a precise mechanism (44). In the light of results from the present study, it now seems very likely that the activation mechanism described by Koch and colleagues is driven mainly by heptose metabolites, presumably ADP-heptose.

In our present work, we tested the activation signature by *H. pylori* and purified bacterial ADP-heptose using the Thp-1 human monocyte-macrophage-like cell line (naïve or pre-differentiated). *In vivo*, professional phagocytes can come into close contact with *H. pylori* in the stomach mucosa and induce cytokine production, inflammation, and induced neutrophil immigration (71) when exposed to the bacteria. In our present study, non-pre-activated Thp-1 cells differentiated upon *H. pylori* or free ADP-heptose co-incubation into a macrophage-like, adherent cell phenotype, a process, which was starting around 8 h after the initiation of the co-incubation. At later time points post-differentiation and in an MOI-dependent manner, the cells started to produce and release IL-8 and IL-1β. This response was indeed significantly different whether *H.*

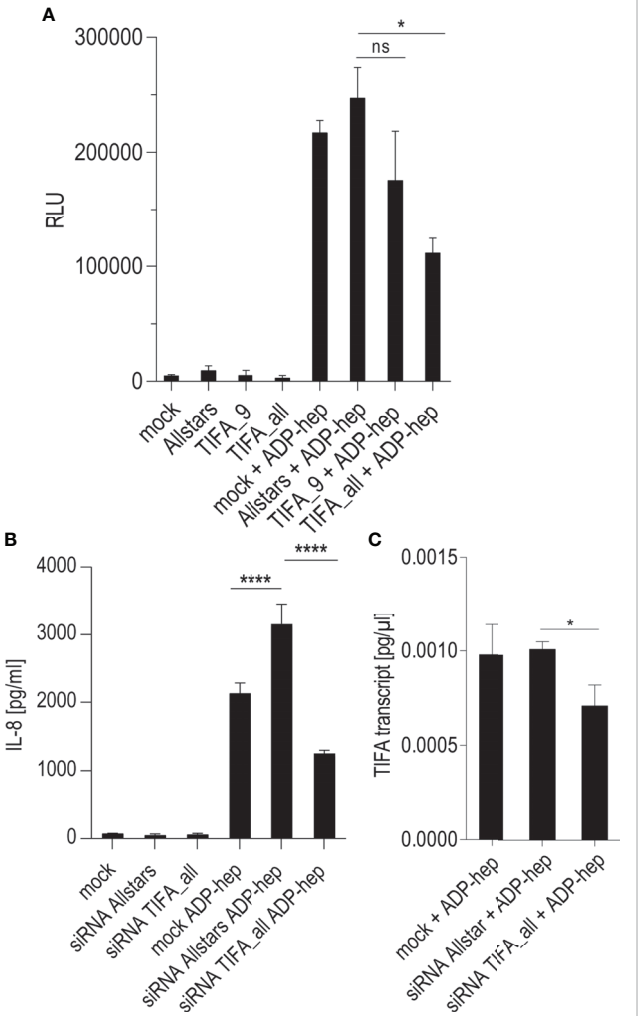

FIGURE 5 | siRNA knock-down of TIFA reveals its important role in activation of Thp-1 cells by ADP-heptose. Panels **(A–C)** show the results of siRNA treatment of Thp-1 cells. **(A)** shows the quantitation of TIFA siRNA effects on NF-κB-dependent luciferase reporter activity (Thp1_luc) after stimulation with 2.5 µM pure ADP-heptose (samples with ADP-hep). Either one single siRNA (TIFA_9) was used, with a moderate but significant reducing effect on activation, and a combination of four siRNAs (see *Methods*) showed a strongly significant effect on NF-κB activation by pure ADP-heptose. Not heptose-stimulated controls of the mock-transfected or siRNA-treated cells are shown alongside (bars on left side), with very little detectable NF-κB activity. Mean and standard error of four independent biological replicates are depicted for each condition. Panel **(B)** shows the reduction of IL-8 release into cell supernatants upon TIFA siRNA knock-down (combination of four TIFA siRNAs) of Thp-1 cells treated with pure ADP-heptose (ADP-hep, 2.5 µM), in comparison to the Allstars negative control siRNA and a mock-transfected sample without siRNA. Same conditions incubated without ADP-heptose are shown on the left side, with no significant activation. Mean and standard errors of six replicates for each condition are shown. In **(C)**, the significant reduction of TIFA transcript in TIFA siRNA-transfected cells in comparison to Allstars siRNA-treated cells and mock-transfected cells (all under co-incubation conditions with ADP-heptose, 2.5 µM, ADP-hep) was verified by qPCR, normalized to human GAPDH transcript, in triplicate samples. Statistical differences were calculated by unpaired student's *t*-test. Significant p values: ****$p < 0.001$; *$p < 0.05$; ns is non-significant.

pylori wild type or heptose mutants or *cag*PAI mutants were co-incubated. Hence, in this setting, LPS heptose metabolite production, partially aided by translocation of the metabolites into the Thp-1 cells *via* the T4SS, was a major driver of cytokine (IL-8) release and NF-κB activation, but not of IFN-γ release. Heptose-dependent cell activation by pure ADP-heptose and *H. pylori* bacteria was also confirmed for primary human monocytes and monocyte-derived macrophages, but the details need further clarification. The relative contribution of the active CagT4SS was not as strong as in co-incubation experiments with human gastric epithelial cells (16, 17), and also correlated with the bacterial MOI used. CagA was not involved in the activation of Thp-1 cells in our present settings, but an active CagT4SS was nevertheless important. Previous results gathered in mouse macrophages (44) did not emphasize an important role of the CagT4SS in macrophage activation. However, in addition to potential differences between mouse and human macrophages in response to heptose metabolites, which needs to be tested in future studies, this divergent result from our study may also be explained by the use of different *H. pylori* strains or of higher MOIs (50 bacteria per cell) in their study as opposed to rather low MOIs which we were testing here (5 to 25 bacteria per cell). Congruently with their study in mouse cells (44), and with the characterization of human macrophages in *H. pylori*-infected patients (72), we collected some evidence that *H. pylori* differentiates human monocyte-like cells towards a proinflammatory M1 macrophage phenotype, or a hybrid M1/M2 phenotype (62–64), which was partially dependent on bacterial heptose biosynthesis. IRF8 transcript upregulation, a marker for macrophage maturation, was also induced here by heptose coincubation. Phagocytosis during short-term exposure of Thp-1 cells up to 24 h was not affected by the presence of pure heptose metabolite. By knocking down TIFA in monocytic Thp-1 cells, we confirmed the important role of TIFA in response to heptose metabolites in this cell type.

Using bacterial mutants, purified bacterial lysates and pure ADP heptose, we determined for the first time that the bacterial LPS inner core metabolite ADP-heptose can be taken up by human monocyte/macrophages in the absence of a dedicated bacterial secretion/injection system. Comprehensive transcript analyses demonstrated that a human gene encoding a monosaccharide transporter of unknown specificity, SLC2A6 (Glut6), which seems to be highly relevant in macrophages (73), was strongly upregulated by heptose exposure. This result might earmark SLC2A6 as a potential importer of heptoses in macrophage-like cells. Interestingly, SLC2A6 was reported recently to be an upregulation marker for the M1 polarization of macrophages (74). In a similar manner as pure ADP-heptose, ETLs generated from *H. pylori* wild type strain and isogenic CagT4SS-deficient mutants activated the Thp-1 cells, as long as ADP-heptose biosynthesis was intact. Cytochalasin D treatment did not reduce the activation by and uptake of pure metabolite to a major extent; this finding supports the possibility of an active transport/import mechanism for heptose metabolites into the cells, as opposed to a primarily random phagocytic mechanism of uptake. ETLs

generated from *H. pylori* ADP-heptose biosynthesis deficient mutants (*hldE*), in contrast, were much less able to induce IL-8 secretion, or transcript, or NF-κB activation, in Thp-1 cells. Conditioned bacterial growth media from ADP-heptose proficient bacteria, with or without a functional CagT4SS, were also able, albeit to a lesser extent, to induce monocyte/macrophage IL-8 production. This result indicates that *H. pylori* grown in the absence of cells seem to release only low amounts of heptose metabolites.

Our genome-wide transcript analyses revealed a strong influence of pure ADP-heptose on the global human transcriptome of Thp-1 cells. Signature transcripts of Thp-1 activated by ADP-heptose at early time points encompassed il-8 (cxcl8), ccl2, ccl3, ccl4, and il-1β, but not il-6 or il-10. Central activation markers of both, ADP-heptose and *H. pylori* wild type bacteria, comprised increased transcript amounts of genes coding for NF-κB transcription factors and coactivators, complement factors, and genes involved in intracellular recognition of double-stranded RNA of the OAS family (65). We detected a substantial overlap of about 350 transcripts regulated by pure ADP-heptose or live *H. pylori* wild type bacteria. Transcription factor NF-κB gene transcript (NFKB2) was very strongly induced by both ADP-heptose and wild type *H. pylori*, but less so by the *hldE* mutant in these settings. In addition, subsets of specific transcripts were differentially regulated by free ADP-heptose or live *H. pylori*. One of the most striking differences was the downregulation of PYCARD/ASC transcript for live *H. pylori* as compared to mock, and the upregulation of the same transcript for the ADP-heptose-treated cells over mock-treated. This effect and transcripts in the same cluster of regulation merit to be followed up in future work. In direct comparisons, live *H. pylori* wild type bacteria and isogenic LPS heptose mutants were rather distinct in their activation of transcripts, although both also showed some transcript overlap.

Despite the identified differences, we determined that transcriptional regulation and NF-κB activation overlapped between Thp-1 cells co-incubated with ADP-heptose, *H. pylori* wild type bacteria or isogenic *hldE* mutants. Since TLR2 was confirmed to be highly expressed in these phagocytic cells and the canonical TLR2 ligand PAM3Cys-SK4, used as a control stimulus in our experiments, activated the cells very strongly, we assume that TLR2 ligands present in the *hldE* mutant bacteria and its lysate preparations contribute to the observed signaling overlap, *via* the TLR pathway. Co-culture with *H. pylori* and *H. pylori*-isolated DNA can activate neutrophil IL-8 production *via* TLR9 (11). However, we did not see a substantial activation of the non-pre-primed Thp-1 cells by *H. pylori* DNA, or by NOD1, NOD2 ligands. Therefore, we surmise that TLR9, NOD1, and NOD2 signaling are rather weak in these settings. NOX production was not observed in Thp-1 cells under the conditions and settings used in the present study (own unpublished data) and was therefore not considered to be affected by heptose. NLRP3 activity seemed to play a minor or no role in the heptose-mediated innate cell activation of these monocyte/macrophages. Earlier work reported that soluble molecules extracted from *H. pylori* are able to activate phagocytic cells to produce IL-8 (14). This seems to be in tune with our present results that heptose production and treated lysates of *H. pylori* bacteria enriched in metabolites can induce proinflammatory signaling, NF-κB activation and IL-8 secretion in Thp-1, and human primary monocytes and monocyte-derived macrophages, very likely *via* the ALPK1-TIFA axis. This might be partially supported by phagocytic or other uptake activities that those cell types exhibit, since cytochalasin D treatment partially inhibited the activation.

Other cells that should be studied for the influence of *H. pylori* heptose-ALPK1-TIFA signaling in the context of *H. pylori* are indeed neutrophils and dendritic cells, which infiltrate the gastric tissue during human *H. pylori* infection (75). In *H. pylori*-infected neutrophils, NLRP3 was previously established as pattern recognition receptor with a major influence (76). Others have reported that CagT4SS-impaired wild type strains and specific CagT4SS mutants (VirB4, VirD4) induced somewhat higher amounts of IL-1β, and less IL-10 in neutrophils than CagT4SS-competent wild type bacteria (77), which might indicate a different role of heptose signaling in those cells.

Taken together, *H. pylori* activates phagocytic cells/macrophages at early time points in a specific manner. This effect is primarily *cag*PAI- and heptose-dependent, if naïve monocyte-like cells come into contact with *H. pylori*. This might apply to the natural habitat in the stomach, where *H. pylori*-naïve monocytes or other phagocytic cells may immigrate or reside in the local tissues. LPS inner core heptose metabolites, most likely including ADP-heptose (21), can be one major activating factor for such early activation and priming *in vivo*. Pure ADP-heptose exerted a comparable effect as wild type bacteria in our assays, both in time course and in strength. For macrophages in direct contact with live *H. pylori* bacteria, activation relied on metabolite transport by an active CagT4SS, which can be provided only by live bacteria. Upon release of heptose metabolites (ADP-heptose), which can occur by different means such as by spontaneous bacterial lysis *in vivo*, induced lysis, or phagocytosis, monocyte/macrophages are also able to take up heptose metabolite without the activity of the CagT4SS or live bacteria. Very likely, other Gram-negative pathogenic bacteria invading different body sites, such as the respiratory, reproductive or intestinal tract, can also activate phagocytic cells by LPS heptose metabolites, in the presence or absence of dedicated bacterial secretion systems. Remaining questions, for example with regard to the role of these interactions and responses for the chronic inflammation and disease settings *in vivo*, or for the interaction with other professional phagocytes and antigen-presenting cells are important subjects to be investigated in further work. A prophylactic and therapeutic vaccine would be a much-desired tool to combat *H. pylori*-mediated diseases and cancerogenesis, but its successful design seems to be continuously hampered by *H. pylori* immune evasive and modulatory mechanisms. For these reasons, in depth studies

about the specific interaction of *H. pylori* with various phagocytic cell types are urgently needed.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA685657.

AUTHOR CONTRIBUTION

LF and SCS contributed to the design of the study, performed and interpreted experiments, and co-authored the paper. MH, MG and MA performed and interpreted experiments and provided materials. CJ conceived the study, performed and interpreted experiments, acquired funding, and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.632154/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplements to Publication I:

Faass et al., Frontiers Immunology 2021

Supplemental materials accessible via:

https://www.frontiersin.org/articles/10.3389/fimmu.2021.632154/full#supplementary-material

Files:

Presentation 1.pdf (see below)

Data Sheet 1.xlsx (large supplemental file Table S1, contained on USB device)

Data Sheet 2.xlsx (large supplemental file Table S2, contained on USB device)

Supplementary Information
Supplementary Figures

Contribution of heptose metabolites and the *cag* Pathogenicity Island to the activation of monocytes/macrophages by *Helicobacter pylori*

L. Faass, S.C. Stein, M. Hauke, M. Gapp, M. Albanese & C. Josenhans

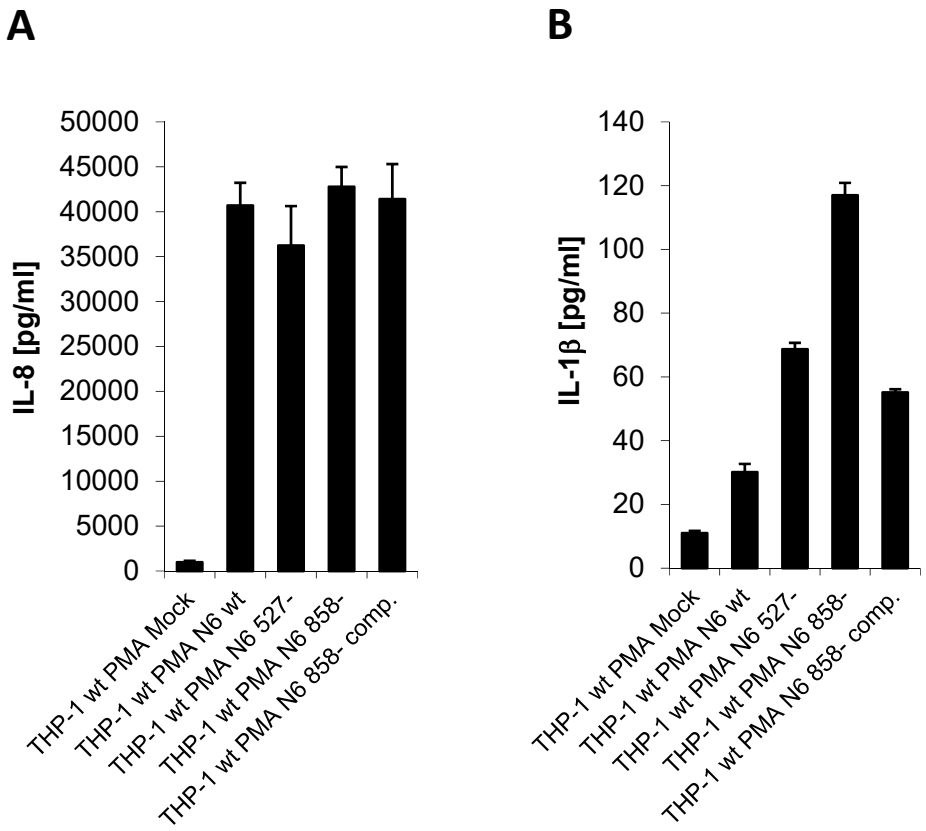

Fig. S1: heptose biosynthesis and the CagT4SS have no direct role in the capacity of live *H. pylori* to induce IL-8 or IL-1β secretion by PMA-preactivated human monocyte/macrophage cell Thp-1. Thp-1 cells which were primed and pre-activated by PMA (see methods), and only afterwards co-incubated with live *H. pylori* (post co-incubation for 4 h). Cytokines in the cell supernatants were quantitated by ELISA **A)** IL-8 secretion; **B)** IL-1β secretion. MOI was set at 25 bacteria per cell. In addition to parental wild type bacteria of strain N6, *cagY* (527-, T4SS functioanlly deficient), *hldE* (858- , core heptose biosynthesis-deficient), and *hldE*-complemented bacteria (858- comp.) were tested.

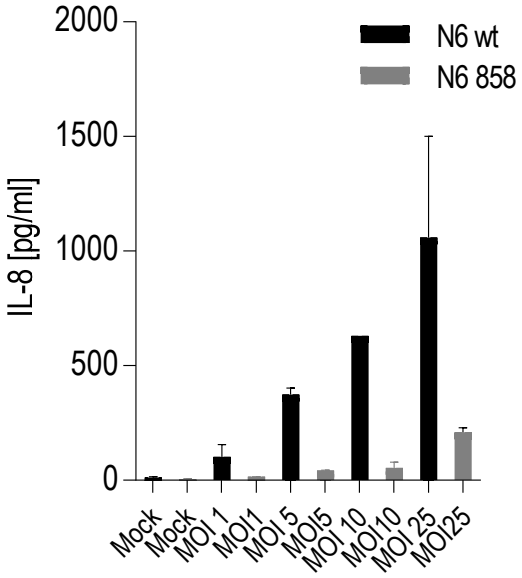

Fig. S2: cell activation in monocyte-like Thp-1 cells upon co-incubation with live *H. pylori* bacteria are influenced by bacterial MOI. Thp-1 cells were co-incubated with *H. pylori* N6 wild type N6 bacteria or isogenic *hldE* mutant bacteria at different MOIs; 20 h post-co-incubation, IL-8 cytokine secretion into the supernatants was determined by ELISA.

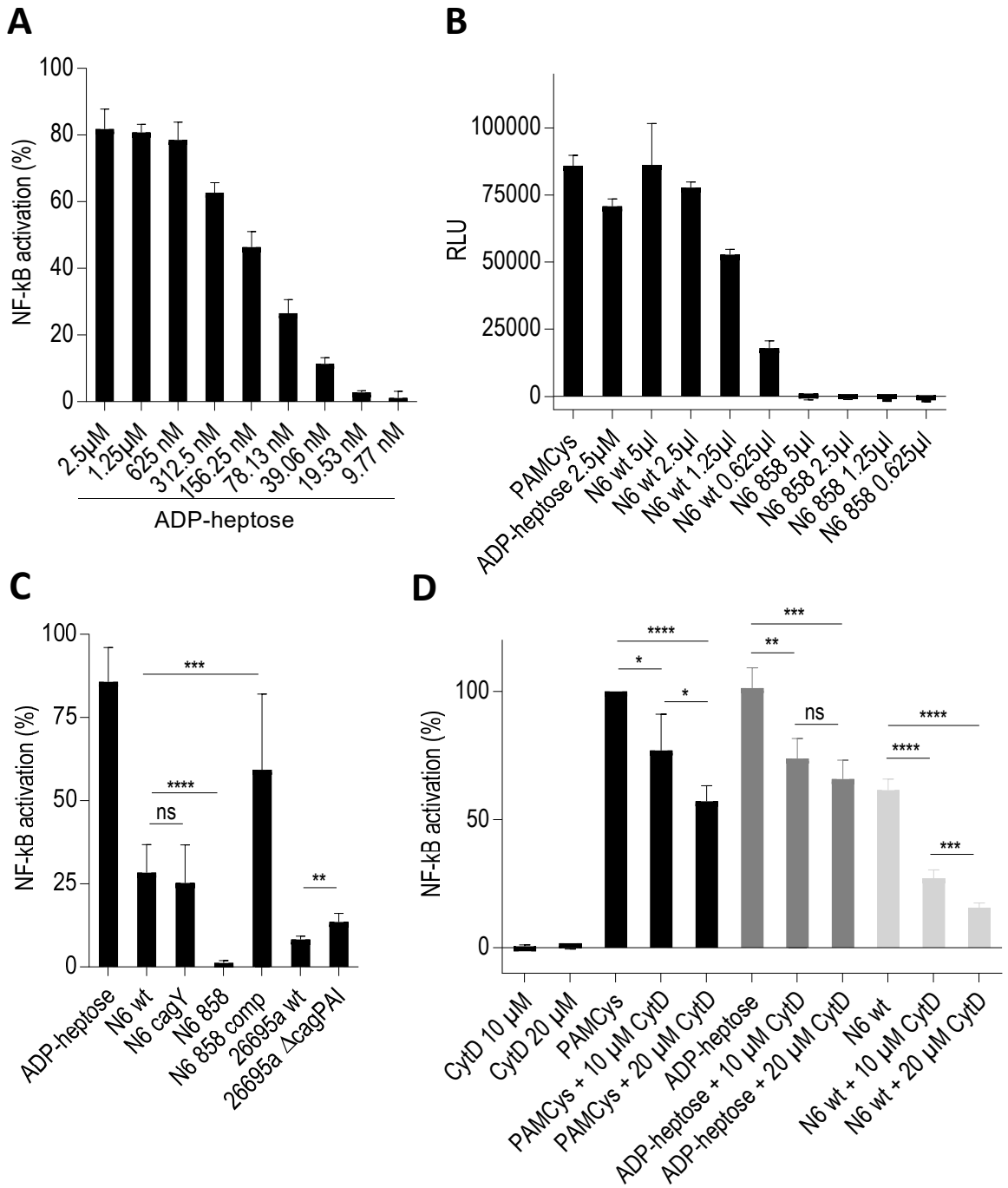

Fig. S3: Characteristics of NF-κB activation in Thp-1 cells by pure ADP-heptose, *H. pylori* enzyme-treated lysates (ETL) or bacterial culture supernatants. A) concentration-dependent activation of NF-κB in Thp1_luc reporter cells by pure ADP-heptose. ADP-heptose was co-incubated with the cells at 5 µM for 4 h. For each concentration, NF-κB activation in %, relative to a PAMCys control (PAMCys) which was set to 100%, is depicted. **B)** Metabolite-enriched ETLs generated from *H. pylori* N6 wild type strain and its isogenic *hldE* mutant (at different volumes as indicated, 96 well plates) were applied to Thp1_luc reporter cells for 4 h. Arbitrary luminescence units (RLU) are shown for each condition. **C)** Culture **s**upernatants generated from liquid cultures of two different *H. pylori* strains as indicated and respective isogenic mutants in the *cag*PAI or heptose biosynthesis (*hldE-*, 858), (20 µl of supernatants per well in 96-well plates) were co-incubated with Thp1_luc reporter cells for 4 h (for strain descriptions see Table 1). Quantitation of luciferase activity is shown for each condition in percent of the positive control PAMCys (20 ng/50 µl), which was set to 100%. **D)** role of cytochalasin D (cytoskeleton inhibitor) in activation of Thp-1 cells (NF-κB) by pure ADP-heptose and live *H. pylori* bacteria. Thp1_luc reporter cells were co-incubated with pure ADP-heptose or live bacteria (strain N6, MOI of 5 bacteria per cell) for 4 h, in the presence or absence of the cytoskeleton inhibitor cytochalasin D (CytD). All values in D) are depicted in % of the positive reference (PAMCys, 100%). Statistical differences in **C**) and **D**) were calculated by unpaired student's *t*-test. Significant p values: ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; ns is non significant. Mock values (very low) were subtracted as background in all assays from A) through D) and are therefore not depicted.

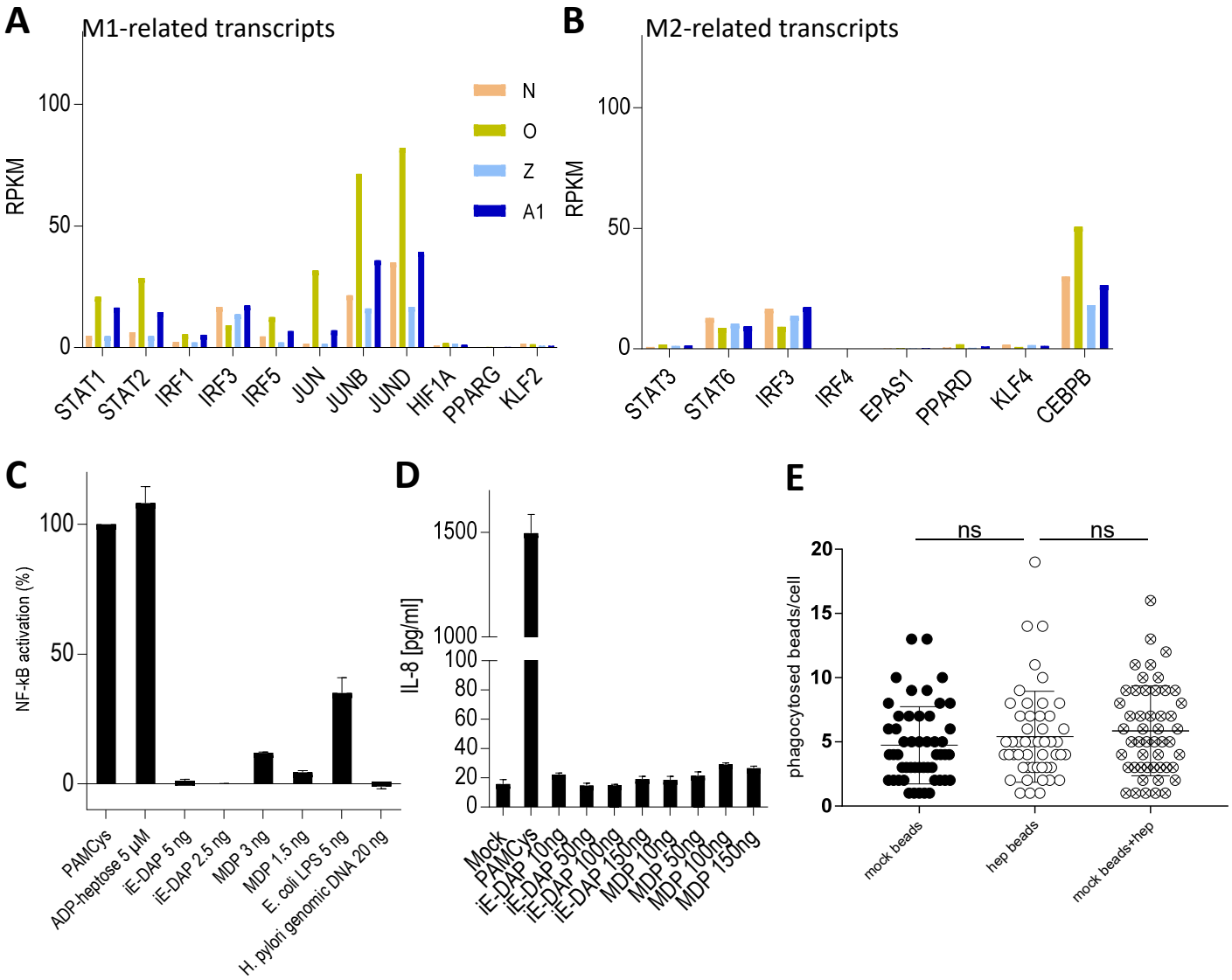

Fig. S4: Selected transcriptome results of Thp-1 cells for macrophage phenotype and contribution of various pattern recognition receptors and their ligands to Thp-1 activation and phagocytosis. assessing the presence and differential expression of transcripts involved in macrophage polarization. In panels **A**) and **B**), we assessed subpanels of transcripts (RPKM) involved in M1 (panel **A**) or M2 (panel **B**) macrophage polarization for differential expression in our comprehensive transcriptome datasets. N, O, Z, A1 designate mock1, *H. pylori* N6 wild type, mock2 and pure ADP-heptose co-incubation conditions with Thp-1 cells, respectively. Compare also Table 3. main Figure 3, and supplementary tables for transcriptome results. Detailed methods for transcriptome results and analyses can be found in the Methods' description. **C**) and D) testing for the activity of NOD1, NOD2, TLR4 and TLR9 ligands in comparison with PAMCys (positive control for activation) and ADP-heptose for NF-κB activation (**C**) or IL-8 secretion (**D)** in Thp-1 cells. In **C**), relative luminescence in % of the PAMCys control is depicted for NF-κB-dependent luciferase activation of Thp1_luc reporter cells (96 well, co-incubation for 4 h) are shown; mock values were subtracted as background). In **D**), IL-8 secretion into the supernatants of co-incubated Thp-1 cells (20 h p.c.) was quantitated by ELISA. PAMCys was applied in C), and D) as a control condition for NF-κB activation. **E**) phagocytosis by Thp-1 cells, co-incubated with fluorescent microbeads (1 µm diameter, $4x10^6$ beads/well in 24 well plate – 20 beads per cell) in the absence or presence of the MAMP ADP-heptose (at 2.5 µM). Ingested beads per cell were counted in fluorescence microscopy. Mock beads: Thp-1 cells co-incubated with beads for 4 h in the absence of innate stimulus; hep beads: Thp-1 pre-incubated with pure ADP-heptose for 16 h, then microbeads were added and co-incubated for another 4 h; mock beads+hep: Thp-1 cells were co-incubated with microbeads and ADP-heptose for 4 h. 50 cells were counted for each condition. Pairwise and multiple comparisons of statistically significant differences in bead uptake (shown on the y-axis) were performed using two-way ANOVA. ns is non significant.

Faass, Stein et al., Suppl. Fig. S4

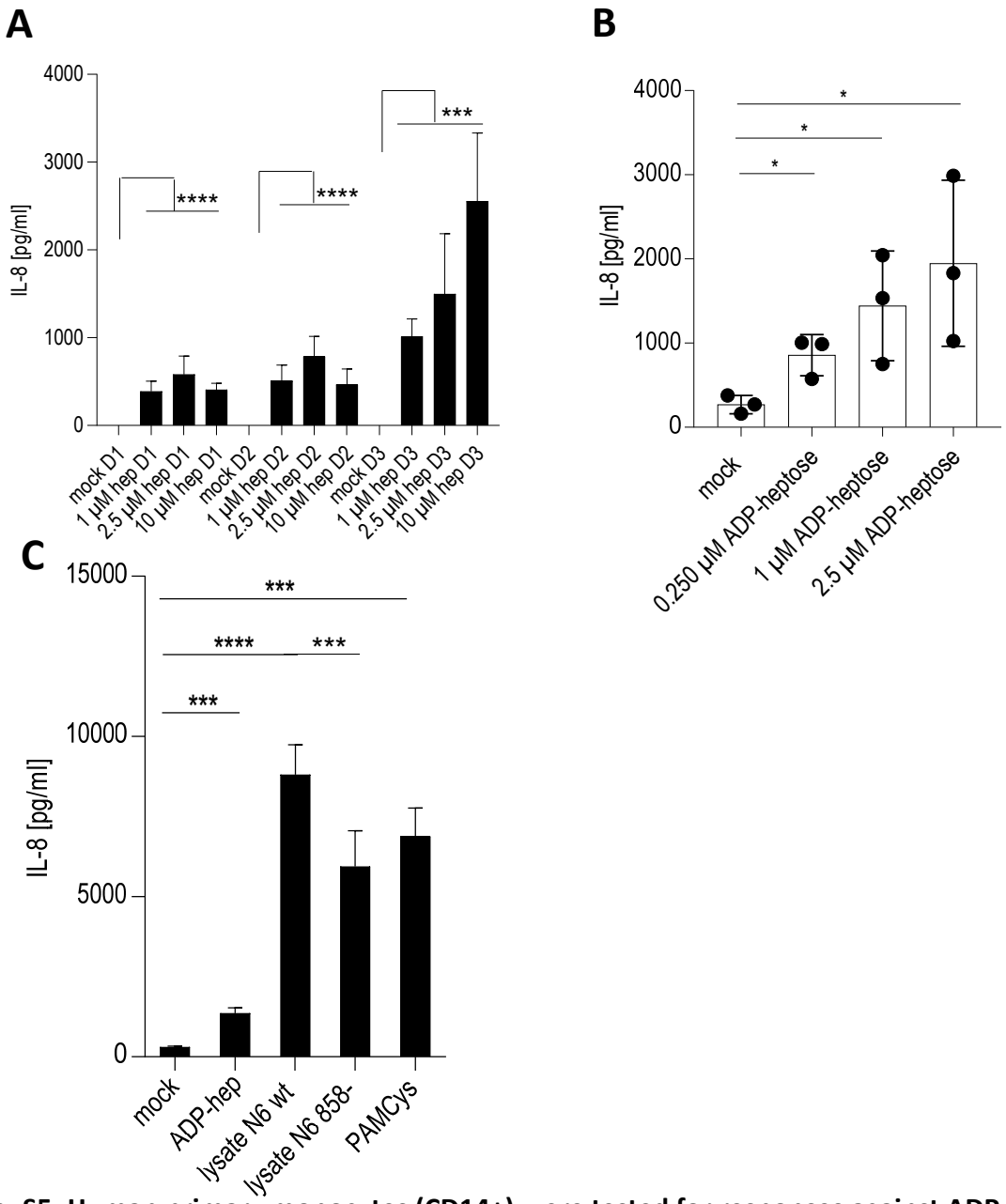

Fig. S5: Human primary monocytes (CD14+) were tested for responses against ADP-heptose and *H. pylori* bacteria. CD14+ cells were isolated and either co-incubated directly with ADP-heptose (hep) or differentiated to hMDMs, subsequently co-incubated with ADP-heptose or *H. pylori* enzyme-treated lysates **A**) Response of non-differentiated CD14+ human primary monocytes (2 x 10^5 cells/well in 24-well plate) from blood PBMC after exposure to pure ADP-heptose (hep) at indicated concentrations, for 20 h. CD14+ monocytes from three independent individual healthy donors (D1, D2, D3) were investigated. **B**) Concentration-dependent activation of differentiated CD14+ human primary monocyte-derived macrophages (2x 10^5 cells/well in 24-well plate) from three independent donors by pure ADP-heptose; All donors are combined in one bar for each condition; mean and standard error for the independent donors are shown for each condition. Despite some between-donor variation (see black ball-shaped symbols), the concentration-dependent activation is clearly visible and significant. **C**) Response of differentiated primary monocyte-derived macrophages (one donor) towards ADP-heptose (ADP-hep, 2.5 µM) or bacterial treated lysates enriched in small metabolites (50 µl pf ETL per ml cell medium in 24 well plates; co-incubation time 4 h). N6 is wild type of *H. pylori* strain N6; 858- is the isogenic *hldE* mutant of strain N6. PAMCys (400 ng/ml) was used as a positive control condition for cell activation. IL-8 secretion into the cell supernatants in **A**), **B**) and **C**) was quantitated by ELISA. Statistically significant differences between mock- and ADP-heptose- or bacteria-co-incubated cells were calculated using two-way ANOVA and are annotated as significant differences to the mock condition (in **A** and **B**) or differences between different co-incubation conditions in **C**); ***$p < 0.001$, ****$p < 0.0001$.

Faass, Stein et al., Suppl. Fig. S5

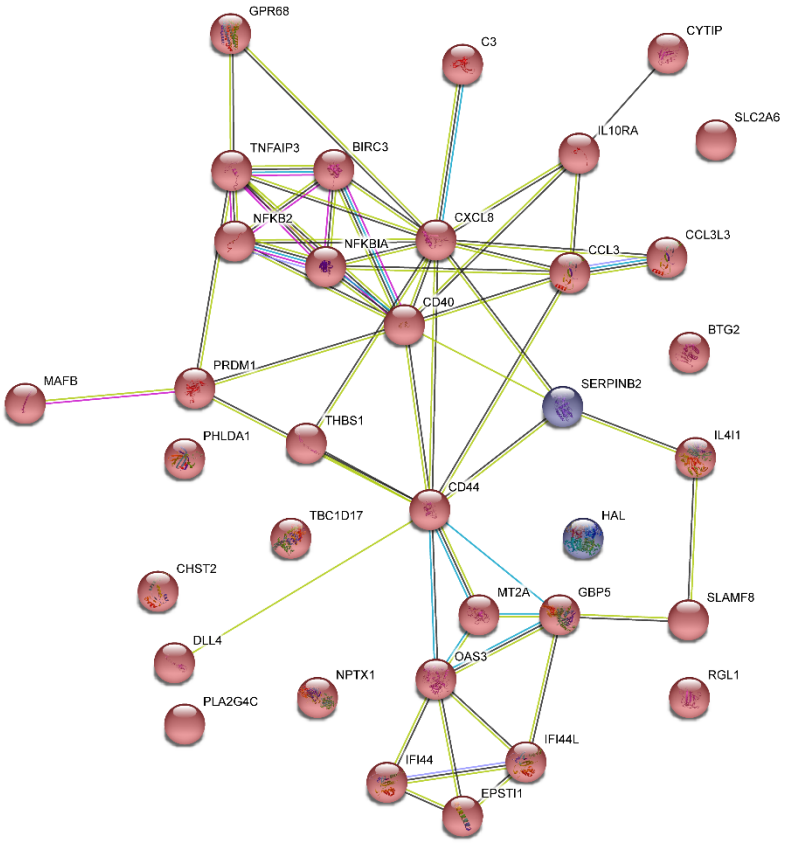

Fig. S6: Results of transcriptome sequencing: pathway analysis and visualization by STRING of transcripts differentially regulated in Thp-1 cells co-incubated with *H. pylori* N6 wild type bacteria or with isogenic *hldE* mutant bacteria. Comprehensive transcriptomes generated from mock-co-incubated and bacteria-co-incubated Thp-1 cells were compared with each other for differential transcript regulation. Subsequently the overlap of differential transcript regulation between wild type bacteria vs. mock (control condition) and *hldE*-mutant bacteria versus mock, respectively, was determined (see Venn digaram in main Fig. 3), and the genes in the intersection of both RNA-seq pairings were analyzed by STRING (stringent cut-off of 8-fold regulated). Commonly upregulated transcripts by the two conditions are shown colored in red, commonly downregulated transcripts are colored in blue. Upregulated genes show a substantial overlap between paired conditions. Some central nodes of activation correspond to main Fig. 3 panel O (STRING diagram), shown for cell incubation with pure heptose versus mock-co-incubated and related to NF-kB activation. Full results are contained in Supplementary Table S2.

3.2 Publication II

Helicobacter pylori Modulates Heptose Metabolite Biosynthesis and Heptose-Dependent Innate Immune Host Cell Activation by Multiple Mechanisms.

Hauke M, Metz F, Rapp J, Faass L, Bats SH, Radziej S, Link H, Eisenreich W, Josenhans C.

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Contribution to Publication II:

First author:

- Conceptual contributions/experimental design
- Investigation: Performing and developing of experiments, evaluation of results, formal analysis and statistics
- Establishing and improving methodology
- Publication: writing of original draft, figures and figure design, references, contributions to final review and editing

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RESEARCH ARTICLE

Helicobacter pylori Modulates Heptose Metabolite Biosynthesis and Heptose-Dependent Innate Immune Host Cell Activation by Multiple Mechanisms

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ABSTRACT Heptose metabolites including ADP-D-glycero-β-D-manno-heptose (ADP-heptose) are involved in bacterial lipopolysaccharide and cell envelope biosynthesis. Recently, heptoses were also identified to have potent proinflammatory activity on human cells as novel microbe-associated molecular patterns. The gastric pathogenic bacterium *Helicobacter pylori* produces heptose metabolites, which it transports into human cells through its Cag type 4 secretion system. Using *H. pylori* as a model, we have addressed the question of how proinflammatory ADP-heptose biosynthesis can be regulated by bacteria. We have characterized the interstrain variability and regulation of heptose biosynthesis genes and the modulation of heptose metabolite production by *H. pylori*, which impact cell-autonomous proinflammatory human cell activation. HldE, a central enzyme of heptose metabolite biosynthesis, showed strong sequence variability between strains and was also variably expressed between strains. Amounts of gene transcripts in the *hldE* gene cluster displayed intrastrain and interstrain differences, were modulated by host cell contact and the presence of the *cag* pathogenicity island, and were affected by carbon starvation regulator A (CsrA). We reconstituted four steps of the *H. pylori* lipopolysaccharide (LPS) heptose biosynthetic pathway *in vitro* using recombinant purified GmhA, HldE, and GmhB proteins. On the basis of one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry, the structures of major reaction products were identified as β-D-ADP-heptose and β-heptose-1-monophosphate. A proinflammatory heptose-monophosphate variant was also identified for the first time as a novel cell-active product in *H. pylori* bacteria. Separate purified HldE subdomains and variant HldE allowed us to uncover additional strain variation in generating heptose metabolites.

IMPORTANCE Bacterial heptose metabolites, intermediates of lipopolysaccharide (LPS) biosynthesis, are novel microbe-associated molecular patterns (MAMPs) that activate proinflammatory signaling. In the gastric pathogen *Helicobacter pylori*, heptoses are transferred into host cells by the Cag type IV secretion system, which is also involved in carcinogenesis. Little is known about how *H. pylori*, which is highly strain variable, regulates heptose biosynthesis and downstream host cell activation. We report here that the regulation of proinflammatory heptose production by *H. pylori* is strain specific. Heptose gene cluster activity is modulated by the presence of an active *cag* pathogenicity island (*cag*PAI), contact with human cells, and the carbon starvation regulator A. Reconstitution with purified biosynthesis enzymes and purified bacterial lysates allowed us to biochemically characterize heptose pathway products, identifying a heptose-monophosphate variant as a novel proinflammatory metabolite. These findings emphasize that the bacteria use heptose biosynthesis to fine-tune inflammation and also highlight opportunities to mine the heptose biosynthesis pathway as a potential therapeutic target against infection, inflammation, and cancer.

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KEYWORDS ADP-heptose, ALPK1-TIFA, *Helicobacter pylori*, mass spectrometry, NMR, bacterial metabolites, heptose-monophosphate, innate immunity, lipopolysaccharide

Heptose derivatives, including ADP-D-glycero-β-D-manno-heptose (ADP-heptose), are produced by Gram-negative and Gram-positive bacteria as building blocks for the biosynthesis of lipopolysaccharide (LPS) structures and other envelope components, such as surface layers (1). In particular, most Gram-negative bacteria require heptose sugars in the inner core of their LPS. Recently, (ADP)-heptose metabolites were also defined as a class of novel microbe-associated molecular patterns (MAMPs) produced by Gram-negative bacteria. Via pattern recognition, these heptose metabolites, in particular ADP-heptose, can be recognized inside mammalian cells and lead to downstream NF-κB activation through the ALPK1-TIFA axis and the formation of tumor necrosis factor receptor-associated factor (TRAF)-interacting proteins with forkhead-associated domain complexes (TIFAsomes) (2–4). This innate cell activation mechanism was already described for a number of pathogenic bacteria, including *Neisseria gonorrhoeae* (5, 6), *Yersinia enterocolitica* (4), diverse *Escherichia coli* strains (4), *Shigella flexneri* (2), *Campylobacter jejuni* (7), and *Helicobacter pylori* (3, 8–12). While some bacteria release the heptose metabolites directly into the medium (7, 13), other species use the targeted properties of complex bacterial membrane secretion systems to inject some of these metabolites into the cytoplasm of mammalian host cells. For example, *H. pylori* appears to use mainly its Cag type 4 secretion system (CagT4SS) to also mediate the transfer of heptoses into human gastric epithelial cells (3, 10, 11) and monocytes/macrophages (12), while *Enterobacteriaceae* (*Shigella* and *Yersinia*) and *Pseudomonas* spp. may also enlist their type 3 secretion systems (T3SS) for a similar purpose (14). Hence, similar to T3SS, which can transport both proteins and metabolites for host cell targeting, this also seems to apply for T4SS.

The core heptose biosynthesis pathway leading to primary production of heptoses is present in most Gram-negative bacteria and also exists in some Gram-positive bacteria (15, 16). Several Gram-negative bacteria use heptoses in their LPS inner core and in their LPS outer core or outer chains (*O*-antigen chains) (1, 17–19). Various bacteria also incorporate heptose sugars into their surface layers or outer polysaccharide capsules (1, 16, 20–24) and into bacterial surface-associated proteins (25). Glycosylation with glycero-manno-heptose was, for instance, detected in the AIDA-I outer membrane-associated autotransporter adhesin of intestinal pathogenic enteroaggregative *E. coli* (25). The canonical biosynthesis pathway of LPS core heptoses leading to nucleotide (ADP)-activated heptose was first reported in *E. coli*, *Haemophilus influenzae*, and *Aneurinibacillus thermoaerophilus* (15, 16, 26–28). GDP-heptose, in addition to ADP-heptose metabolites, has also been identified, for instance, in *Mycobacterium tuberculosis* and enteropathogenic *Yersinia enterocolitica* (29, 30). The canonical heptose biosynthesis pathway generally consists of five steps, which are mediated by the consecutive action of four or five different enzymes, for instance, in *E. coli* and *H. pylori*, GmhA, HldE (RfaE), GmhB, and HldD (RfaD) (16, 31, 32). ADP-activated heptoses, delivered by the last biosynthesis steps, can then serve as substrates for the downstream glycosyltransferases involved, for example, in the assembly of the LPS inner core oligosaccharides (33, 34).

Despite the long-standing knowledge about the canonical heptose biosynthesis pathway, very little is known about the regulation of the biosynthetic gene cluster, the diverse enzymatic activity profiles of the contributing enzymes, and the roles of various heptose biosynthesis genes in different bacteria and strain variability of those traits in different bacterial species. The gene cluster and single gene or enzyme activities have been characterized most intensively in *E. coli* (15, 28, 31, 32, 35). *H. pylori*, the model organism that is studied here, contains short heptane chains with α-configured D-glycero-D-manno-heptose units in its LPS core (19, 36). In addition, some, preferentially non-Asian strains assemble branched heptose subunits in their outer core (19). *H. pylori* harbors one single canonical heptose biosynthesis gene cluster, consisting of *gmhA*/*rfaA* (HP0857), a bifunctional bidomain-encoding *hldE*/*rfaE* gene (HP0858), as in *E. coli*,

and *gmhB* (HP0860) and *hldD* genes (HP0859). In *H. pylori*, the individual enzymes encoded in the core heptose biosynthesis cluster were putatively assigned, and the enzymatic activities of these proteins were partially reported (8, 37). D-Glycero-β-D-manno-heptose-1,7-bisphosphate (β-HBP), a major reaction product in *Neisseria* (5), and ADP-heptose, produced by *H. pylori*, *Y. enterocolitica*, *Salmonella*, and *E. coli*, were shown to exhibit an innate activation potential toward human cells via the ALKP1-TIFA axis (4, 8).

In the case of guided host cell activation by the secretion system of pathogenic bacteria (for example, by transport or translocation of heptose metabolites into human cells), the important question arises of whether and how the bacteria have developed ways to cell contact-dependently and strain-dependently control the biosynthesis of phospho-heptose derivatives and their innate activity on target cells. To address these open questions, we have studied *H. pylori*, which directs heptose metabolites into human cells mainly by means of its T4SS (3, 11, 12). First, we tested the regulation of genes in the heptose biosynthesis cluster in various strains and assessed the influence of several physiological parameters on the regulation of heptose biosynthesis in *H. pylori*. Using this approach, we have identified that strain identity, the presence of a functional CagT4SS, or the allelic diversity of the biosynthesis genes themselves contributed to strain- and condition-dependent modulation of heptose biosynthesis and metabolite activity on host cells. By reconstituting the pathway *in vitro* and characterizing final and intermediate products *in vitro* and in *H. pylori* lysates biochemically, we have also gained insight into the variation of known and novel output metabolites (products) of the heptose pathway and their activity as novel MAMPs.

RESULTS

Strain-specific sequence diversity, differential regulation of genes, and protein expression in the *H. pylori* LPS heptose biosynthesis gene cluster. To form a basis for assessing the heptose biosynthesis capacities in *H. pylori* and their strain-dependent genetic diversity, we initially analyzed the genomic organization of the heptose biosynthesis gene cluster and sequence polymorphisms of its cluster genes in different *H. pylori* strains. The *H. pylori* heptose biosynthesis gene cluster, similar to the *C. jejuni* cluster (7), has a counterintuitive organization, which is conserved in all *H. pylori* strains; the genes that are relevant in the later steps of the biosynthesis pathway are transcribed first and are located directly downstream of the nontranslated 3′ region. The *gmhA* gene (HP0857), which encodes the first enzyme in the pathway, is the last gene of the cluster (Fig. S1A in the supplemental material). *hldE* (HP0858), the second to last gene in the operon, encodes a bifunctional enzyme with two domains (d1 and d2) that are required for two separate steps in the biosynthesis pathway (Fig. 1A; Fig. S1A). Interestingly, the *H. pylori hldE* gene displays a relatively high sequence diversity between strains, while the other genes of the cluster and their predicted protein products are highly conserved (Fig. S1B), as expected for a housekeeping function. As a basis for further functional investigations, we sought to clarify fundamental questions regarding the transcript quantities of the *H. pylori* heptose biosynthesis cluster genes and how those genes might be regulated. First, we determined the transcript amounts within the gene cluster for strain N6. This is the only strain where we could obtain insertion mutants in all heptose cluster genes (11). While the first genes in the cluster, presumably expressed from a housekeeping sigma^{80} promoter (38), had comparably low transcript amounts, the downstream genes in the cluster, *hldE* (HP0858) and *gmhA* (HP0857), displayed much higher transcript levels (Fig. 1B). This cannot be explained by the known transcript start sites identified in reference strain 26695 (39), where no additional start sites (primary or processed) are located directly upstream of HP0858 in the heptose gene cluster. Genome-wide transcriptome analyses revealed that the respective transcripts are strongly regulated by growth phase and by 5-methylcytosine (m^5C) DNA methylation (40 and own unpublished data). We next addressed further internal and external influences on gene regulation and possible regulation mechanisms.

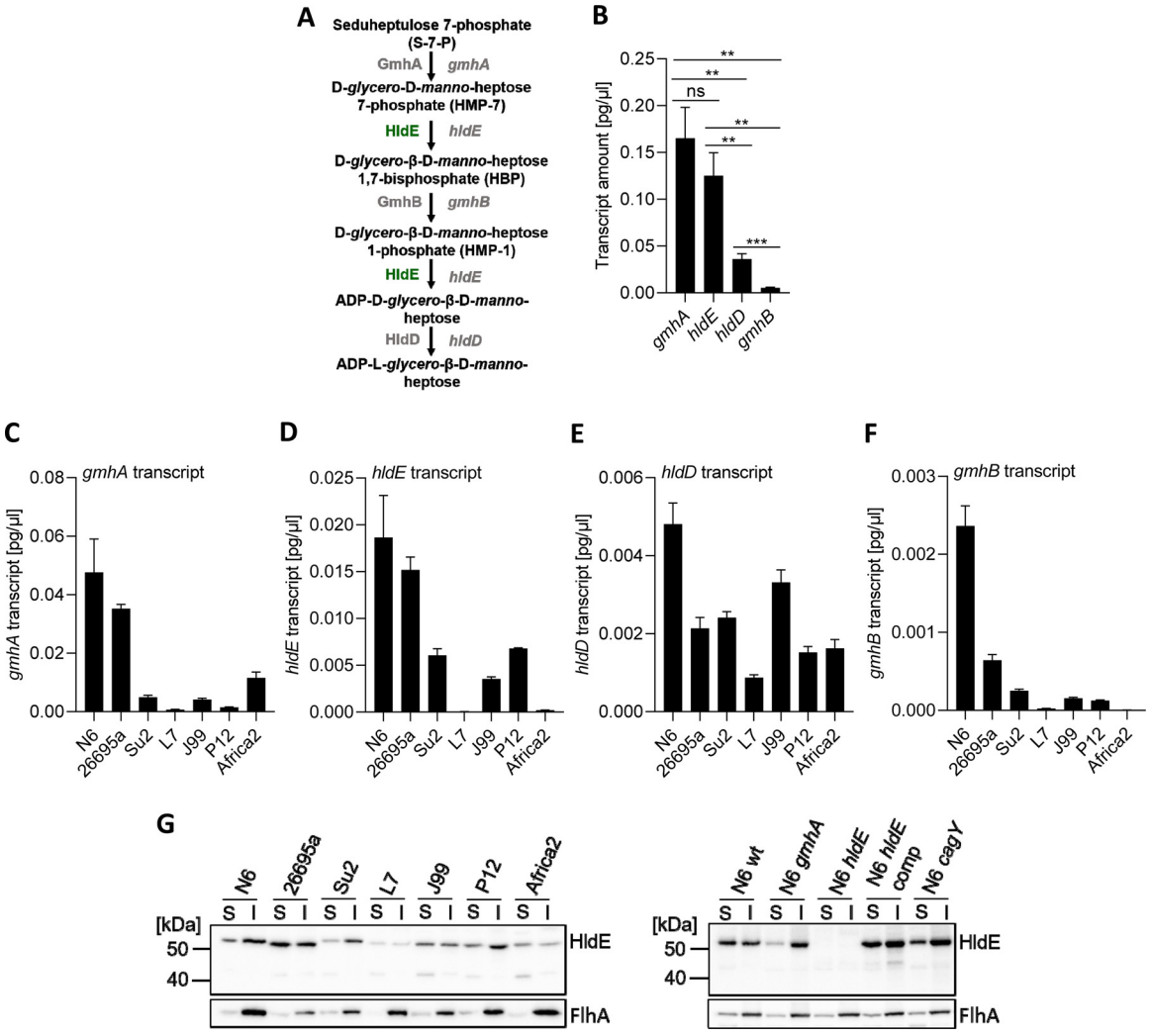

FIG 1 Expression of heptose biosynthesis cluster genes and gene product HldE in *H. pylori* is gene specific and strain specific. (A) Schematic of the *H. pylori* 5-step ADP-L-heptose biosynthesis pathway with enzymes and proposed intermediate reaction products. The central enzyme HldE/RfaE (green) is encoded by the gene HP0858 (*hldE*) and is a bifunctional enzyme performing two steps of the synthesis cascade. (B) Quantification of absolute transcript amounts of each transcript of heptose biosynthesis cluster genes *gmhA*, *hldE* (HP0858), *hldD*, and *gmhB* on *H. pylori* strain N6 cDNA, as determined by RT-qPCR; ns, not significant. (C to F) Diverse *H. pylori* wild-type strains of different geographical origins were used to compare the transcript amounts of genes *gmhA* (C), *hldE* (D), (*hldD*/*rfaD*) (E), and (*gmhB*) (F) between different strains. All RT-qPCR assays were performed in technical triplicates. The results are given as absolute values for transcript amounts normalized with a correction factor to 16S rRNA transcript amounts for each strain. Strain N6 was used as a reference for normalization. (G) Immunoblot (anti-HldE antiserum, 1:20,000) detecting the bifunctional protein HldE and the loading and fractionation control protein FlhA (flagellar membrane protein) in soluble (S) and insoluble (I) fractions of different *H. pylori* wild-type strains (left) and of selected isogenic mutants (right) of the *H. pylori* strain N6. Ten micrograms of total protein was loaded in each lane to provide equalized amounts; wt, wild-type. Comparisons for statistically significant differences in B were performed between all conditions using an unpaired Student's *t* test; significant *P* values are marked with asterisks; **, $P < 0.01$; ***, $P < 0.001$. Statistics for all pairwise comparisons in C to F were performed by two-way analysis of variance (ANOVA), followed by a Tukey *post hoc* test and are summarized in Table S1 in the supplemental material.

Expression of LPS heptose biosynthesis genes and proinflammatory host cell activation are *H. pylori* strain specific. Earlier work (41) established that *cag*-positive, live *H. pylori* strains can induce very different activation levels in gastric epithelial cells at early time points, which can now be attributed almost exclusively to heptose metabolite signaling via the ALPK1-TIFA pathway (2, 11). Hence, we asked the question of what differences in heptose biosynthesis (genetic or metabolite output) between strains might exist that can be responsible for the strain-variable activation potential.

We first tested seven different *H. pylori* strains from different geographical locations and bacterial populations (41) (Table 1) for their heptose gene cluster transcripts (Fig. 1C to F). Strong strain-specific differences in transcript amounts for each single gene were detected,

TABLE 1 List of strains used in the present study

Strain name	Origin	Description	Reference
H. pylori (HP) N6 wt[a]	hpEurope; site of isolation is France	Wild-type strain, *cag*PAI positive	56
HP N6 *cagY* (HP0527)	hpEurope	Allelic exchange insertion mutant HP0527 (*cagY*) in strain N6	11
HP N6 *hldE* (HP0858)	hpEurope	Allelic exchange insertion mutant HP0858 (*hldE*) in strain N6	11
HP N6 *hldE comp*	hpEurope	HP0858/*hldE* gene complementation in the *rdxA* locus of the HP0858 mutant in strain N6	11
HP N6 *csrA::aphA3′-III*	hpEurope	*csrA* (HP1442) allelic exchange insertion mutant (Materials and Methods) in N6	This study, generated according to reference 43
HP 26695a	hpEurope; site of isolation is United States	Wild-type strain, *cag*PAI positive	57
HP 26695a Δ*cag*PAI	hpEurope	*cag*PAI complete deletion by allelic exchange in strain HP 26695a	11
HP 26695a *cagY*	hpEurope	Allelic exchange insertion mutant HP0527 (*cagY*) in strain HP 26695a	This study, according to reference 11
HP L7 wt	hpAsia2, South Asia, India	Wild-type strain, *cag*PAI positive	41
HP L7 Δ*cag*PAI	hpAsia2, South Asia, India	*cag*PAI complete deletion by allelic exchange in strain L7	This study
HP J99 wt	hpAfrica1, North America	Wild-type strain, *cag*PAI positive	58, 59
HP Africa2 wt	Africa2	Wild-type strain, primary *cag*PAI negative	58
HP Su2 wt	hpNEAfrica, Northeast Africa, Sudan	Wild-type strain, *cag*PAI positive	41
HP Su2 Δ*cag*PAI	hpNEAfrica, Northeast Africa, Sudan	*cag*PAI complete deletion by allelic exchange in strain Su2	This study
HP P12 wt	hpEurope	Wild-type strain, *cag*PAI positive	60
E. coli Rosetta pLysS	Novagen	*E. coli* protein expression strain	Novagen

[a]All wild-type (wt) strains listed are minimally passaged clinical isolates.

with up to two logs of differences in absolute specific transcript quantities between strains (e.g., between N6 and Su2). The highest absolute transcript quantities in most strains, but also strongest quantitative transcript differences between strains, were obtained for transcripts of *gmhA* and *hldE* (Fig. 1C and D). In different strains, divergent patterns of relative transcript levels of heptose gene cluster genes were determined (Fig. S1C to G). According to differences in relative transcript levels of *gmhA*, *hldE*, *gmhB*, and *hldE*, strains can be grouped into two major clusters (A and B), of which, cluster A strains (such as 26695a, N6, and Su2) had the highest expression of both *gmhA* and *hldE* transcripts. In contrast, strains in cluster B (for example, L7 and Africa2, a primary *cag* pathogenicity island [*cag*PAI]-negative isolate) exhibited the highest expression of *gmhA* and *gmhB* (HP0860), whereas the second functional gene of the pathway (*hldE*) was much less expressed (Fig. 1A; Fig. S1A and C to G). This seems to suggest that the strains have evolved distinct patterns concerning enzymatic activities and potential metabolite output of the heptose pathway.

To back up the transcript analysis by other methods focusing on pathway output, we assessed proinflammatory activity of pathway products and compared them with transcript levels of the pathway genes for various strains. Enzymatically treated lysates (ETLs) of the bacteria, as demonstrated previously (11), are a good proxy measure for overall content in proinflammatory cell-active heptose metabolites in the bacteria, when grown independently of cells. In previous work, we had tested the ETLs of only two different wild-type strains (11), which appeared to have comparable proinflammatory MAMP activities, dependent on active heptose biosynthesis, but appeared to be significantly lower than for live bacteria. To clarify this preliminary result, we tested live bacteria and ETLs (treated lysates) generated from seven *H. pylori* wild-type strains (six *cag*PAI-positive strains and one primary *cag*PAI-negative strain) for their activity on AGS cells, MKN28 stomach epithelial cells, and HEK_luc NF-κB-luciferase reporter cells (Fig. 2; Fig. S2). Most strains' ETLs activated proinflammatory cell responses at a comparable low level (Fig. 2A) and similar to the response to a reference amount of β-D-ADP-heptose (the readout was luciferase reporter quantification or interleukin-8 [IL-8] secretion, and the results of an ADP-heptose titration are shown in Fig. S4B). The only exception for ETL activity was strain N6, which

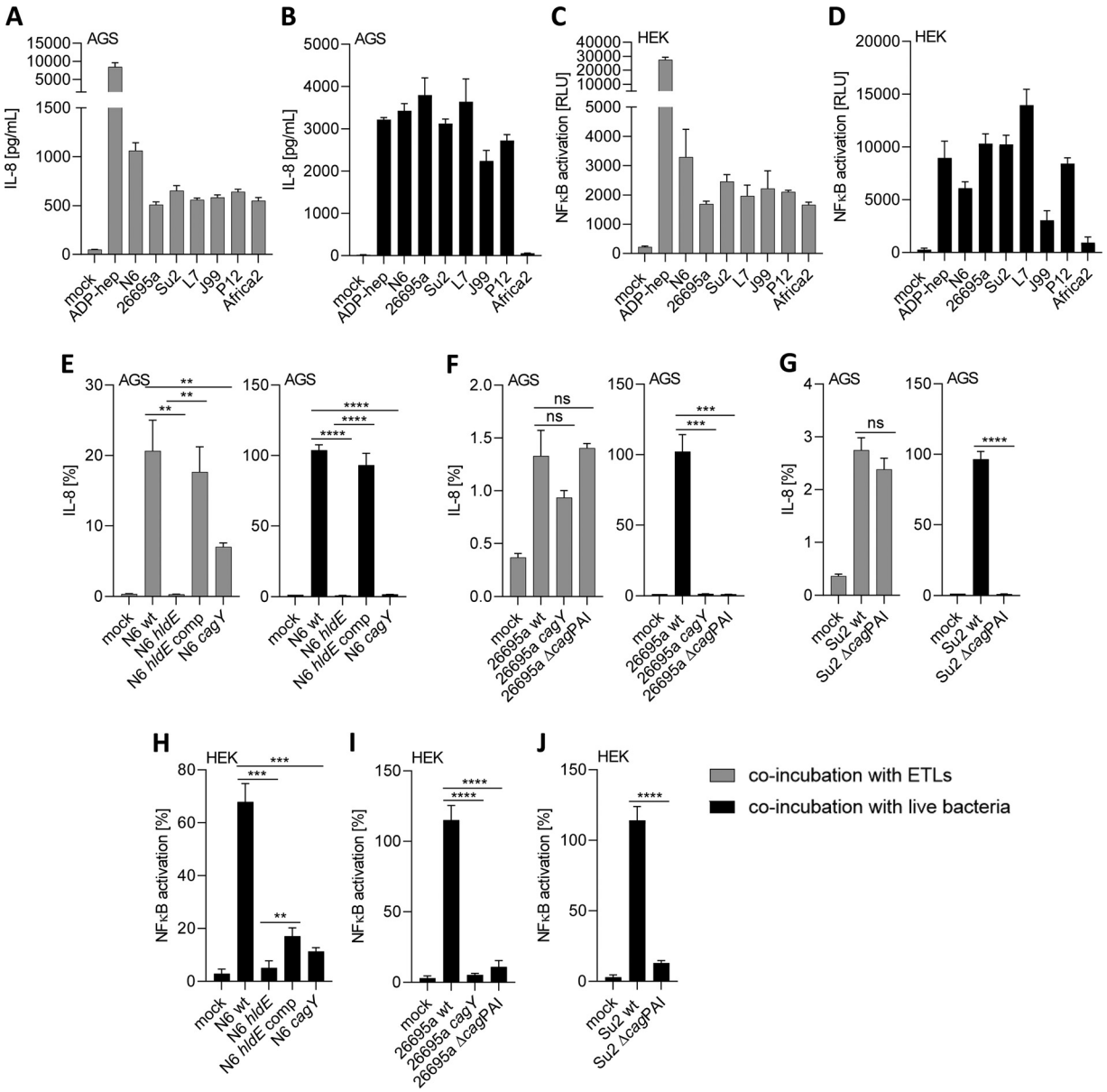

FIG 2 Coincubation of epithelial cells with ETLs from various *H. pylori* strains and corresponding live bacteria demonstrates distinct potential in proinflammatory cell activation. (A to D) Proinflammatory activation of gastric epithelial AGS cells (A and B) or HEK cells (C and D) by ETLs prepared from wild-type strains (A and C) or corresponding live bacteria (B and D) (MOI = 25). Cells were coincubated with ETLs or live bacteria for 4 h; activation was measured by IL-8 ELISA on cell culture supernatants (AGS) or luminescence measurement of the NF-κB-luc reporter cell line (HEK), respectively. Absolute values of the response are depicted for IL-8 (in pg/mL) and for luciferase measurements in relative luminescence units (RLU) as shown on the *y* axes; for comparative purposes, we applied ADP-heptose (2.5 μM) as a control stimulant for each experiment. (E to G) Activation of AGS cells after coincubation with ETLs from different *H. pylori* wild-type and mutant strains or the corresponding live *H. pylori* bacteria (MOI = 25) for 4 h. Cell activation by ETLs produced from *H. pylori* wild-type strains and mutants is shown in gray. AGS cell activation by live bacteria is shown in black. Cell responses were quantitated in each experiment by IL-8 ELISA from cell culture supernatants. Triplicate measurements of biological duplicate experiments are shown. Experiments were repeated at least twice with comparable results. (H to J) Proinflammatory cell activation of HEK-NF-κB_luc reporter cells after coincubation with live *H. pylori* bacteria for 4 h. Cell responses were quantitated by measuring luminescence. All conditions were performed in biological triplicates, and experiments were repeated at least once on different days. The results in E to J are depicted in percent values and were normalized to a ADP-heptose-exposed (at 2.5 μM, not shown) control condition performed in each experiment. Comparisons for statistically significant differences in E to J were performed between wild-type-exposed samples and each mutant-exposed sample using unpaired Student's *t* tests. Significant *P* values are marked with asterisks; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

reproducibly activated cells about 2-fold more by its cleared lysate than the other strains (Fig. 2A), suggesting a higher content in proinflammatory metabolites. Those results indicated an overall low innate activity for preformed heptose pathway products contained in bacteria grown in the absence of cells, in particular as suggested for those metabolites

that can be taken up actively by the target cells (42). The live bacteria, except for the *cag*PAI-deficient strains, all activated NF-κB and elicited IL-8 secretion, with significant interstrain differences in heptose-dependent cell activation (Fig. 2B and D), as previously demonstrated (41). Proinflammatory activation by live bacteria was about 10-fold higher than ETL-mediated activation (IL-8 or NF-κB reporter quantitation) (Fig. 2). The relative proinflammatory activation patterns between the tested ETLs from different strains were comparable for the different epithelial cell lines used (Fig. 2A, 2C; Fig. S2). The results likewise highlighted that live bacteria and the active CagT4SS provide the most important means of targeted cell transport for heptose metabolites in wild-type isolates.

To assess the role of additional bacterial functions for heptose metabolite activity, we generated another set of mutants and compared them with their isogenic wild-type strains. Those included complete *cag*PAI deletion mutants (strains 26695a, Su2, and L7) (see Table 1 for strain list) and in strain N6 (where we could not obtain a *cag*PAI deletion) a *cagY* insertion mutant (defective T4SS). For N6, we also used the previously characterized isogenic *hldE* mutant and an *hldE*-complemented strain as heptose-negative and heptose-positive references, respectively (11) (Table 1). When we generated ETLs from the *H. pylori* isogenic mutants in the heptose pathway and from mutants deficient in T4SS assembly and tested them in comparison to the respective wild-type strain ETLs on various cell types and reporter cell lines, we found a strong difference in epithelial cell activation (Fig. 2A to G), which was entirely dependent on HldE activity and influenced by an active CagT4SS. ETLs from *cagY* mutants had an intermediate phenotype of proinflammatory cell activation on AGS cells, lower than the wild type, while ETLs from Δ*cag*PAI mutants showed no change in proinflammatory activation compared with the wild type. Live bacteria of the same strains and mutants (Fig. 2E to J) exhibited a different activation pattern compared to the ETLs. Live *hldE*-mutant bacteria showed almost no cell activation (as previously published) (11), while live bacteria from *cagY* and Δ*cag*PAI mutants (both T4SS deficient) strain independently activated cells significantly less than their corresponding parental strains. This outcome confirmed that live bacteria generally require an active T4SS for strong innate immune activation of epithelial cells. We next analyzed bacterial lysates of our set of seven diverse *H. pylori* strains by Western blotting (Fig. 1G) using a custom-produced polyclonal antiserum against HldE to detect strain-specific differences in protein expression. The serum was highly reactive against a protein of the predicted mass (~65 kDa) in all tested *H. pylori* strains but not in an *hldE* mutant (Fig. 1G). The serum identified highly variable expression of HldE protein in the different assayed *H. pylori* wild-type strains (Fig. 1G). We detected rather uniform HldE expression in a range of isogenic heptose (11) and *cag* mutants in *H. pylori* N6 (Fig. 1G).

Regulation of heptose biosynthesis pathway transcripts in *H. pylori* is influenced by the absence or presence of the *cag*PAI or contact with human cells. We hypothesized that altered environmental conditions and the presence or functionality of the CagT4SS in *H. pylori* can influence the activity of the heptose biosynthesis pathway, as heptoses will be transported by the secretion system, and, possibly, the T4SS can influence production and identity of cell-active proinflammatory heptose metabolites. Therefore, we tested in a more targeted manner whether heptose gene cluster regulation and the output and activity of heptose reaction products would be modulated by mutation of the *cag*PAI (complete isogenic deletion mutants leading to CagT4SS deficiency) (Fig. 3). We found that complete deletion of the *cag*PAI had a significant reducing effect on transcript amounts, in particular on *hldE* and HP0859 (*hldD*) genes, and had a partially reducing effect on other genes of the cluster (real-time quantitative PCR [RT-qPCR]) (Fig. 3A to C). This observation was independently verified to be similar in three strains (26695a, Su2, and L7). The same pattern of downregulation of heptose biosynthesis transcripts was seen in the transcriptome data of a *cag*PAI deletion mutant compared to the parental wild-type strain (Fig. 3D).

Furthermore, using RT-qPCR, we investigated how bacteria exposed to AGS cells in the presence or absence of a functional CagT4SS regulated and expressed the LPS heptose biosynthesis gene cluster (Fig. 3E). Interestingly, we quantitated a significant upregulation of all genes of the heptose gene cluster in wild-type and *cag*PAI-deficient bacteria that were associated for 4 h with AGS cells. At 8 h of coincubation, the wild-

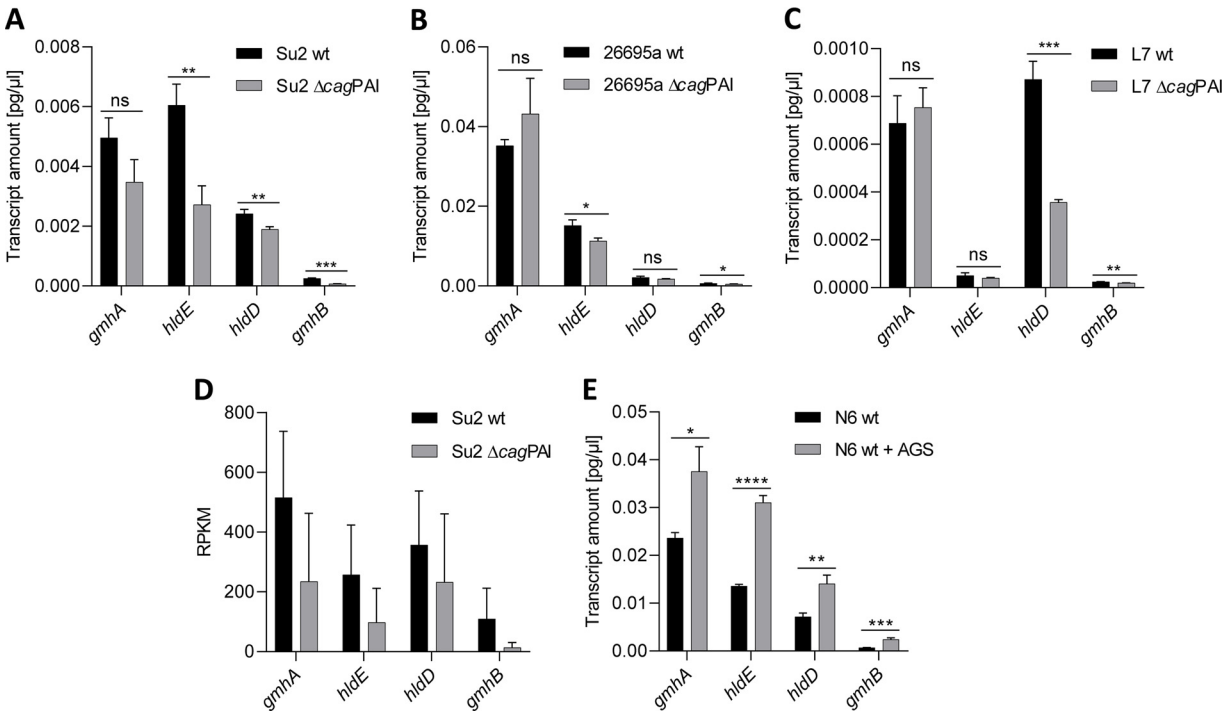

FIG 3 Regulation of heptose pathway transcripts in *H. pylori* is influenced by the presence of the *cag*PAI and contact with human gastric epithelial cells. (A) Transcript quantification (RT-qPCR) of heptose biosynthesis genes of the *H. pylori* Su2 wild-type strain compared with its isogenic Δ*cag*PAI mutant. (B) Transcript quantification (RT-qPCR) of heptose biosynthesis genes HP0857 (*gmhA*) through HP0860 (*gmhB*) of the *H. pylori* 26695a wild-type strain compared with its isogenic Δ*cag*PAI mutant. (C) Transcript quantification (RT-qPCR) of heptose biosynthesis genes of the *H. pylori* L7 wild-type strain compared with its isogenic Δ*cag*PAI mutant. (D) RPKM for genes *gmhA*, *hldE*, *gmhB*, and *hldD* extracted from comprehensive transcriptome data for the Su2 wild-type strain or the Su2 Δ*cag*PAI mutant. (E) Transcript amounts (RT-qPCR) for genes *gmhA*, *hldE*, *hldD*, and *gmhB* of the heptose biosynthesis cluster (Fig. S1 in the supplemental material) of the *H. pylori* N6 wild-type strain coincubated in the presence or absence of AGS cells (MOI = 50) in cell culture medium for 4 h. All RT-qPCR assays were performed in triplicates and normalized against 16S rRNA transcript for each sample. RT-qPCR results in A to C and E are shown as absolute values (pg/μL), using 16S rRNA amounts and the respective wild-type samples as a reference for normalization. Statistically significant differences between conditions were calculated using Student's *t* tests. Significances are marked with asterisks; *, $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$; ****, $P < 0.0001$; ns, not significant.

type strain maintained a stronger upregulation (Fig. S3) than the CagT4SS-deficient mutant strain.

Transcriptome analyses of *H. pylori* in the presence or absence of the *cag*PAI reveal numerous transcript changes partially associated with the carbon starvation regulator CsrA. Bacterial transcriptome changes have the potential to provide a comprehensive perspective on gene expression. Therefore, we performed whole bacterial transcriptome analyses on *H. pylori*, comparing the parental strain with an isogenic *cag*PAI deletion mutant, which we hypothesized would mimic a state of intrabacterial ADP-heptose enrichment by absence of the CagT4SS. Conditions of a "closed" or nonfunctional T4SS, which we showed reduces the expression of heptose genes in three different strains (Fig. 3), can also be helpful in identifying regulators or regulons possibly involved in feedback regulation of heptose biosynthesis. The transcriptome analyses indeed revealed an influence of *cag*PAI deletion on numerous genes of different functional categories (Fig. 4A; Table 2; Table S2). Using a 1.5-fold cutoff, about 500 genes were found to be either up- or downregulated (Table S2). These included genes from different KEGG categories and also genes from the heptose biosynthesis gene cluster, with HP0858/*hldE* and HP0860/*gmhB* genes being significantly downregulated in single differential expression analyses (for information on the complete regulation data set, see Materials and Methods and Table S2). After comparison with known regulons, a number of previously reported CsrA-dependent transcripts in *H. pylori* (compare to the gene table in reference 43), including transcripts of motility- and metabolism-associated genes, were differentially regulated under those conditions (Fig. 4A). CsrA

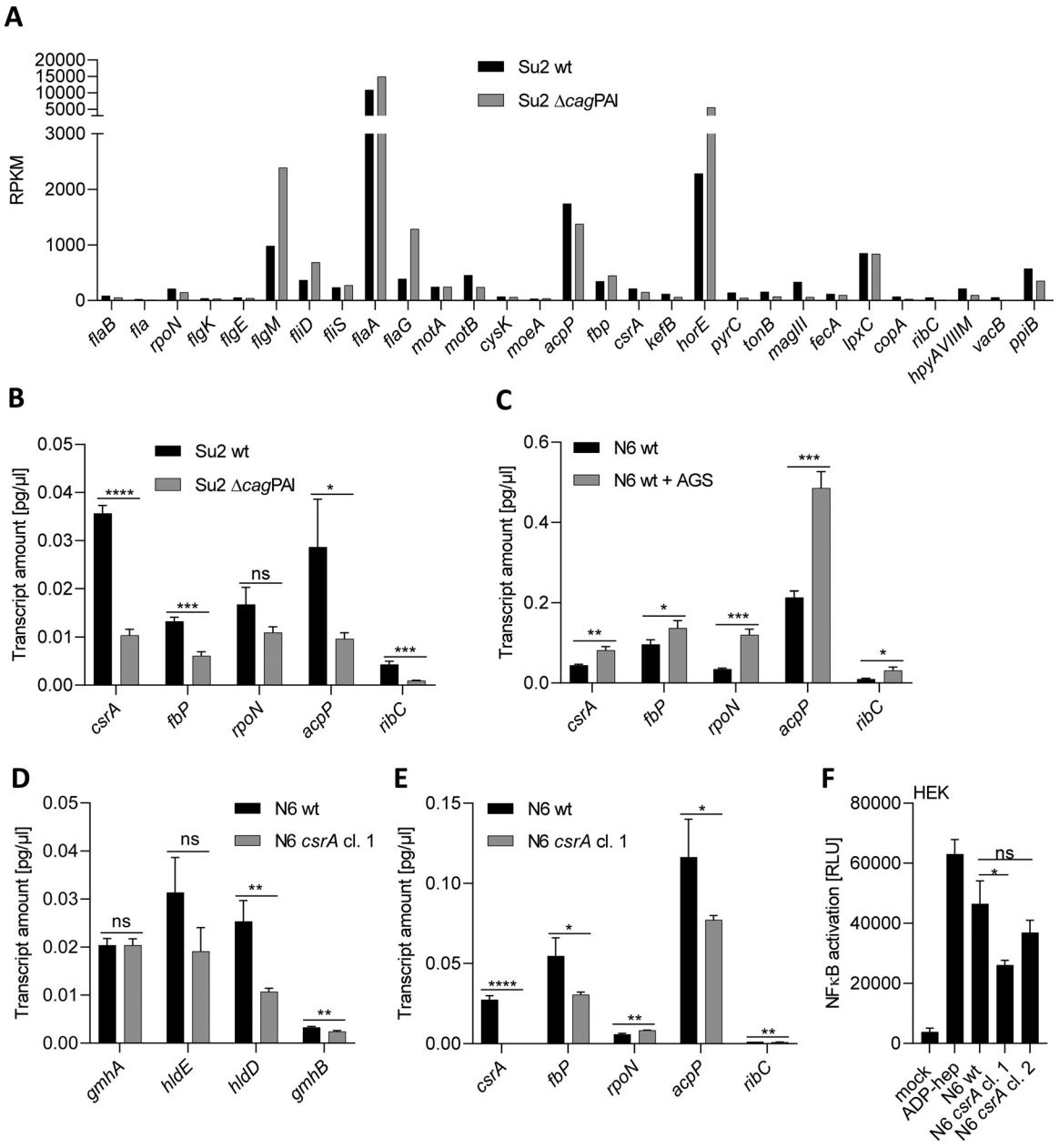

FIG 4 Heptose biosynthesis genes and other functional gene categories in the absence of a functional CagT4SS or in the presence of cells are regulated in association with the carbon starvation regulatory protein CsrA and in *csrA* mutants. (A) Selected RNA-seq results depicted as RPKM values from a transcriptome analysis comparing *H. pylori* wild type (wt), strain Su2, and its isogenic *cag*PAI deletion mutant. A representative experiment (R1) of two independently performed biological replicates is shown. The comparative data of the two replicates are fully listed in Table S2 in the supplemental material. Functionally annotated transcripts that are included in the CsrA regulon as defined in reference 43 are shown, representing functional categories of motility-related or metabolic genes. (B) The same strains cultured under the same conditions were also compared for transcript amounts of CsrA-dependent genes (*csrA*, *fbP*, *rpoN*, *acpP*, and *ribC*) by RT-qPCR. (C) *H. pylori* (strain N6) cultured in the presence or absence of AGS cells was also tested by RT-qPCR for selected CsrA-dependent transcripts as defined under B. (D and E) An *H. pylori* N6 *csrA* mutant (allelic exchange insertion) was generated, and specific gene transcripts of the heptose gene cluster and selected transcripts of the *H. pylori* CsrA regulon as in B and C were tested by RT-qPCR. (F) Reporter assays using HEK_NF-κB luciferase reporter cells (HEK) to detect changed proinflammatory activity of *H. pylori* (strain N6) *csrA* mutants on the heptose-dependent proinflammatory response. Two *csrA*-mutant clones, cl. 1 and cl. 2, were tested alongside the N6 wild-type and reference pure ADP-heptose. Statistically significant differences between conditions were tested by unpaired Student's *t* test. Significances are marked with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $p < 0.0001$; ns, not significant.

broadly shifts metabolism, motility, and other functions in response to environmental conditions in various bacterial species (43–46). Therefore, we also quantitated in detail the selected CsrA-related transcripts *csrA*, fructose-bisphosphatase (*fbp*), riboflavin synthase subunit (*ribF*), *rpoN* (flagellar sigma factor), and acyl carrier protein subunit P

TABLE 2 Part of comprehensive differential expression analysis for transcriptomes of *H. pylori* (strain Su2) in the presence or absence of the *cag*PAI

Gene name[a]	Max group mean	Log_2 fold change	Fold change	*P* value
HP0172 (*moeA*)	34.88	0.79	1.73	0.04
HP0472 (*horE*)	5,524.81	1.96	3.88	0
HP0579	52.42	−2.4	−5.27	6.50E−03
HP0580	67.00	−1.39	−2.62	1.42E−03
HP0581 (*pyrC*)	141.52	−0.91	−1.89	1.01E−03
HP0601 (*flaA*)	14,938.62	1.14	2.2	0
HP0602 (*magIII*)	333.82	−1.77	−3.42	4.91E−10
HP0603	80.49	−1.86	−3.64	2.29E−03
HP0629	54.18	0.95	1.94	8.87E−05
HP0715	556.56	1.71	3.27	0
HP0751 (*flaG*)	1,284.04	2.40	5.29	0
HP0752 (*fliD*)	688.1	1.59	3.01	0
HP0753 (*fliS*)	270.58	0.87	1.82	4.53E−04
HP0754	125.88	−1.07	−2.10	0.08
HP0817	177.73	−1.08	−2.12	6.33E−03
HP0906	38.85	−1.78	−3.43	6.72E−04
HP1051	912.63	−1.88	−3.68	7.77E−16
HP1052 (*lpxC*)	848.04	0.67	1.59	1.58E−06
HP1076	71.56	-0.67	−1.59	0.18
HP1087 (*ribC*)	53.09	−1.32	−2.5	0.01
HP1122 (*flgM*)	2,389.80	1.96	3.89	0
HP1233	62.97	−1.02	−2.03	0.09
HP1248 (*vacB*)	56.09	−2.02	−4.04	1.18E−06
HP1396	164.94	1.02	2.03	4.77E−06
HP1439	13.65	−0.81	−1.75	0.61
HP1566	57.96	−3.70	−12.97	8.50E−03

[a]The subset of the differential expression analysis shows CsrA-dependent genes according to reference 43; only transcripts with a greater than 1.5-fold change (ratio of Su2 Δ*cag*PAI to Su2 wt [reference sample]) are included; a threshold of a maximum group mean of RPKM greater than 10 was applied. Gene name abbreviations are given in parentheses; gene numbers are those of strain 26695 (53). Complete differential expression analyses (samples B13 and B14) are contained in Table S2 in the supplemental material.

(*acpP*) by RT-qPCR comparing expression between wild-type and *cag*PAI-mutant strains and confirmed the significant regulation of most of the transcripts (Fig. 4B) between both conditions. We also compared those selected, differentially regulated, CsrA-dependent transcripts with incubation of bacteria in contact with AGS cells, which impacts the expression of heptose biosynthesis gene cluster transcripts, as shown above (Fig. 3E). Indeed, we found *acpP*, *csrA*, and the CsrA downstream genes *fbp* and *rpoN* to be significantly upregulated in bacteria coincubated with gastric epithelial cells for 4 h (Fig. 4C). Generating a *csrA* mutant, we also verified that the heptose cluster and selected CsrA regulon transcripts are indeed dependent on CsrA function, confirming their differential expression between the parental strain and the *csrA*-mutant strain by RT-qPCR (Fig. 4D and E). As a correlate of heptose biosynthesis with relation to proinflammatory cell activation, we demonstrated that *csrA* mutants show significantly lower activities than parental wild-type bacteria on NF-κB luciferase reporter cells (Fig. 4F), suggesting that not only transcript amounts but also cell-active heptose metabolite MAMP production are reduced in the mutant. Taken together, these findings demonstrated that absence of the T4SS or human cell contact exerted an inversely correlated influence on the bacteria, including regulation of heptose transcripts and other functions, in a CsrA-related manner.

Reconstitution of the *H. pylori* heptose biosynthesis pathway *in vitro* reveals various cell-active proinflammatory reaction products. The results of our regulation analyses and the observed strain differences in heptose cluster transcripts and HldE sequence and expression levels prompted us to investigate which heptose compounds and which quantities of heptose products can be synthesized by the bacteria, resulting from the different reaction steps of the heptose pathway. In addition, transfection of *H. pylori* ETLs into reporter cells versus the medium coincubation of reporter cells with ETLs revealed different responses, suggesting that more than one

heptose product is present (see below). Using mass spectrometry (MS), Pfannkuch and colleagues had so far predominantly found β-D-ADP-heptose (with trace amounts of β-D-heptose-1,7-bisphosphate-β-D-HBP) in treated lysates of *H. pylori* (one strain tested) (8). ADP-heptose is a cell-permeable metabolite (4, 8). β-D-HBP was suggested to be a second cell-active heptose metabolite, possibly produced in a strain-variable manner, which is not cell permeable (8, 42). Until now, other intermediate heptose metabolites of the pathway had not been identified in *H. pylori* or any other bacteria. To approach the question of various products of the *H. pylori* enzymes, we decided to reconstitute the reaction pathway of *H. pylori in vitro* based on the three central, natively purified *H. pylori* enzymes of the pathway (GmhA, HldE, and GmhB) (Fig. 5; Fig. S4) from heterologous expression in *E. coli*. We also expressed and purified both functional domains of the bifunctional HldE enzyme (d1 and d2 domains; see Fig. S1A and Fig. S4) separately for testing. Since the *H. pylori* HldE enzyme, due to its clear sequence heterogeneity between strains, was the eminent candidate for pathway and product heterogeneity within the biosynthesis pathway, we also expressed and purified the full-length enzyme from two different *H. pylori* strains (Fig. 5). We used these proteins in various one-pot *in vitro* reactions to convert seduheptulose-7-phosphate (S7-P) substrate in the presence or absence of ATP (see Materials and Methods). As an immediate readout, we first tested the reaction products of different enzyme compositions on NF-κB luciferase reporter cells (HEK_luc epithelial cells) for determining cell-directed proinflammatory innate activation (Fig. 5A; Fig. S4). Single purified enzymes, incubated without ATP and substrate, and heat-inactivated enzymes were also tested in separate reaction mixtures as controls to exclude potential cell-activating contaminations after purification from *E. coli* (which all gave negative results) (Fig. S4A).

Reaction products of the successful one-pot reconstitution with three main pathway enzymes combined (GmhA, HldE, and GmhB) avidly activated cells (Fig. 5; Table 3; Fig. S4). The concentration dependency of reaction products obtained *in vitro* was also established through titration in comparison with pure β-D-ADP-heptose (Fig. S4B). We also reconstituted a reaction with only two purified enzymes, GmhA and HldE, expecting to obtain mainly β-D-HBP as a reaction product. The reaction product(s) from this second reaction unexpectedly also activated cells strongly without the need for cell transfection (Fig. 5A and C). However, titrations showed that this two-enzyme mixture was about one log less active than the three-enzyme mixture (Fig. 5C). When incubated with nucleotides other than ATP, we obtained proinflammatory product with all nucleotides except dTTP in the three-enzyme mixtures (Fig. 5B).

For HldE enzymes purified recombinantly from two different strains, which show considerable amino acid sequence diversity (Fig. S1B), both produced cell-active proinflammatory compounds. However, the enzymatic action of HldE from N6 resulted in a significantly stronger cell activity in the two-enzyme reaction than HldE from strain 26695 (Fig. 5D). Even for HldE-only reaction mixtures, we were able to produce low-level cell-active output (Fig. 5D). In this case, the resulting activity was almost zero for 26695 HldE and was significantly higher for HldE from N6 strain (Fig. 5D). For all reaction mixtures, we verified whether the resulting activity was dependent on the ALPK1-TIFA pathway by comparing their activity on AGS wild-type and AGS TIFA-knockout (k/o) cells (11) using IL-8 release (enzyme-linked immunosorbent assay [ELISA]) as a readout. The TIFA-k/o cells showed no proinflammatory activation by our mixtures, confirming lack of product activity in the absence of TIFA-mediated signaling, while an alternative innate NF-κB stimulant, *Salmonella* FliC flagellin (TLR5 ligand), was positive (Fig. 5E).

Proinflammatory heptose pathway products identified as β-D-ADP-heptose and β-HMP-1 using NMR and mass spectrometry. To assign compound structures to the one-pot reaction products, we then used ^{1}H NMR analysis of the reaction mixtures containing different sets of recombinant enzymes from *H. pylori* (Fig. 6). Based on the specific chemical shifts, coupling constants, and scalar correlations observed in two-dimensional correlation spectroscopy (COSY) experiments (Table S3), β-D-ADP-heptose

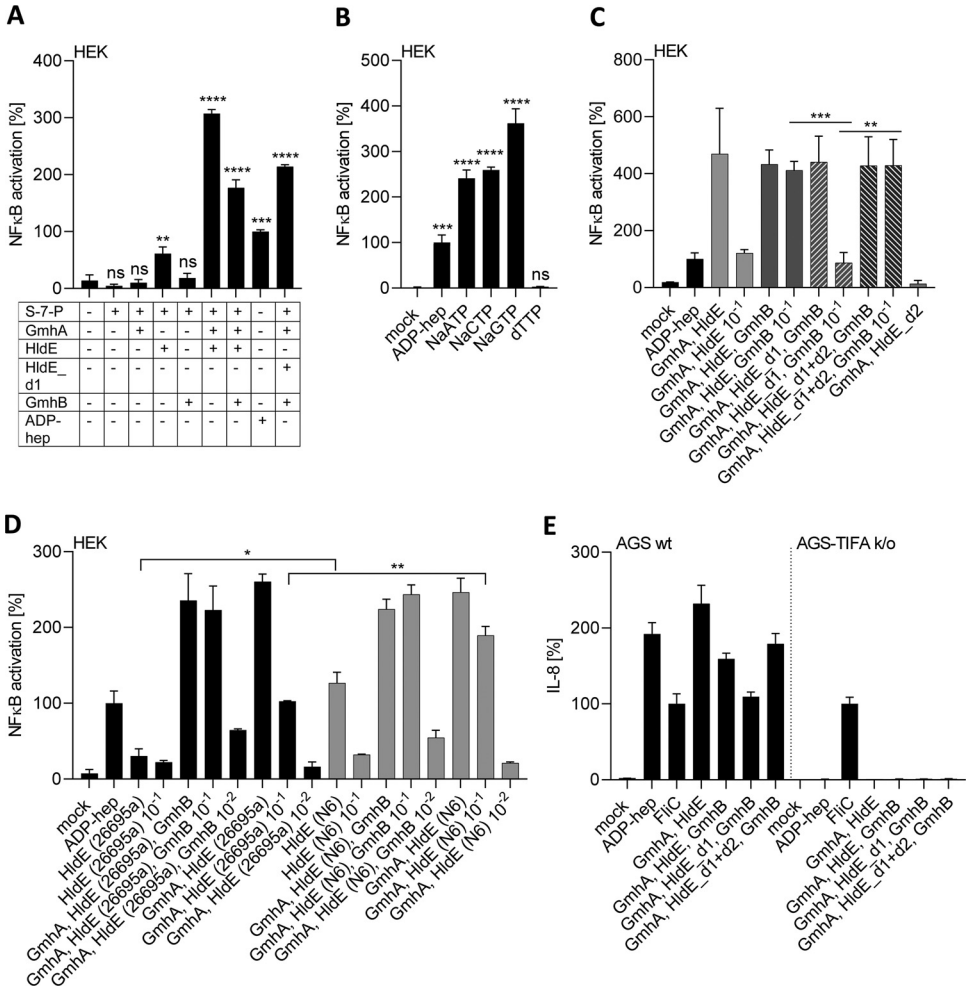

FIG 5 Reconstitution of *H. pylori* ADP-heptose biosynthetic pathway. *In vitro*-synthesized heptose metabolites of distinct enzyme combinations activate epithelial cells to different extents. (A) Proinflammatory activation of NF-κB reporter cells (HEK-NF-κB_luc) by heptose metabolites synthesized *in vitro* using recombinantly purified *H. pylori* proteins (strain 26695a) from substrate seduheptulose-7-phosphate (S-7-P). Absolute luciferase values are shown and are depicted in comparison to pure ADP-heptose (2.5 μM) coincubated cells (control condition). (B) HEK reporter cell NF-κB activation (normalized to 2.5 μM ADP-heptose condition, which was set to 100%) by products *in vitro* synthesized using purified enzymes GmhA, HldE, and GmhB (all from strain 26695a) and different nucleotides (all at 5.8 mM). (C) HEK_luc reporter cell NF-κB activation (normalized to the ADP-heptose control condition, set to 100%) of *in vitro* reaction products synthesized by a combination of GmhA, GmhB plus HldE (the latter as complete protein or as combined, separately purified HldE d1 and d2 domains, HldE_d1+d2), or HldE domain one (HldE_d1, from strain 26695a), or HldE domain two (HldE_d2) purified separately (nondiluted and reaction products diluted 10^{-1} are shown for each condition). (D) NF-κB activation by products synthesized *in vitro* using the three-enzyme combination with highly purified HldE (complete protein) from two different *H. pylori* strains, 26695a or N6, as indicated on the *y* axis (GmhA and GmhB remain in all reactions of strain 26695a). Strongly active mixtures were added nondiluted, at a dilution of 10^{-1}, or at a dilution of 10^{-2}. The data from the reporter cell assays in A to D were summarized from three biological replicates. (E) Proinflammatory activation of AGS wild-type (wt) and AGS-TIFA-knockout (k/o) cells by *in vitro*-synthesized heptose products, quantitated using IL-8 ELISAs on cell supernatants. Values are shown in percent and were normalized to the activation level of *Salmonella* FliC activation control (TLR5 ligand, 400 ng/well, set to 100%). The results shown in E were summarized from two biological replicates for each experimental condition, each quantitated in technical triplicates. Statistical evaluation of significant differences was performed using unpaired Student's *t* tests and shows statistically significant differences. Nonsignificant differences are not indicated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

was identified as the main reaction product of the combined action of GmhA, HldE, and GmhB proteins when ATP was supplied as the energizing nucleotide (Fig. 6G). Comparing with reference compounds (β-D-ADP-heptose and β-L-ADP-heptose; confirmed by NMR) (Fig. 6E and F), we showed that the ADP-heptose product of the *H. pylori* enzyme reconstruction reaction was of the D-form (ADP-D-glycero-β-D-manno-heptose) (Fig. 6G). In the two-enzyme reaction (GmhA and HldE), NMR spectrometry

TABLE 3 Sample identifiers for NMR and mass spectrometry analyses and identification and quantification (chromatogram peak heights) of heptose metabolites detected by LC-ESI MS/MS in *in vitro* reconstitution mixtures and bacterial lysate (ETL) samples

Sample ID	Sample name (description)	Peak height ADP-heptose	RT ADP-heptose (min)[b]	Peak height HBP (ambiguous RT)	Peak height HMP-7/S7-P	RT HMP-7/S7-P (min)[b]	Peak height HMP-1	RT HMP-1 (min)[b]
In vitro reconstitution mixtures[a]								
P0015_S7	9 (GmhA, HldE, GmhB)	5,657,222	10.44	1,848	NP	NP	1,022,031	8.74
P0015_S8	11 (GmhA, HldE)	144,298	10.41	33,115	710,533	8.54	NP	NP
P0015_S9	9d1 (GmhA, HldE_d1, GmhB)	74,731	10.41	2,636	NP	NP	1,561,142	8.74
P0015_S10	11d1 (GmhA, HldE_d1)	12,537	10.40	48,309	836,843	8.54	NP	NP
P0015_S11	11N6 (GmhA, N6_HldE)	346,331	10.39	4,251	1,546,445	8.54	NP	NP
P0015_S12	GmhA only	5,476	10.41	1,723	1,884,683	8.55	NP	NP
P0015_S13	HldE only	1,641	10.40	1,181	2,136,561	8.54	NP	NP
							Peak height HMPv	
H. pylori lysates (ETLs)								
P0015_S14	N6 wt 1 (replicate 1)	14,189	10.42	1,239.96	837	8.70	421.46	8.80762
P0015_S15	N6 *gmhA* (11)	318	10.47	ND	768	8.68	ND	ND
P0015_S16	N6 *hldE* (11)	192	10.40	ND	649	8.68	ND	ND
P0015_S17	N6 *hldE* comp (11)	7,858	10.42	ND	650	8.67	340.96	8.80762
P0015_S18	N6 *gmhB* (11)	217	10.40	ND	697	8.69	ND	ND
P0015_S19	26695a wt 1 (replicate 1) (11)	7,943	10.41	ND	474	8.65	399.94	8.80762
P0015_S40	N6 wt 2 (replicate 2)	15,091	10.29	—	—	—	—	—
P0015_S42	26695a wt 2 (replicate 2) (11)	5,926	10.29	—	—	—	—	—
P0015_S45	Su2 wt	8,928	10.29	—	—	—	—	—
P0015_S47	L7 wt	3,600	10.29	—	—	—	—	—
P0015_S49	Africa2	3,137	10.28	—	—	—	—	—
P0015_S50	J99 wt	5,385	10.30	—	—	—	—	—
P0015_S51	P12 wt	31,530	10.29	—	—	—	—	—
Reference compounds								
Ref_ADP-Heptose (1 μM)		17,208	10.40	NP	NP	NP	NP	NP
S7-P (2.5 μM) pHILIC		NP	NP	NP	49,455	8.56	NP	NP

[a]*In vitro* reconstitution mixtures (Materials and Methods) contain purified enzymes from *H. pylori* strain 26695a if not indicated otherwise.
[b]RT, retention time; NP, not present; ND, clear peak not detectable; —, not measured.

identified another, unexpected, cell-activating product, which was not identical to β-D-HBP but was identified as predominantly β-heptose-monophosphate-1 (β-HMP-1), in addition to the reaction mixture ingredients (Fig. 6H). Dilution titration of the two-enzyme reaction mixture containing β-HMP-1 yielded about 10-fold lower activity on cells (without transfection) than the three-enzyme reconstitution (Fig. S4B). Since previous studies had suggested that β-D-HBP is preferentially produced by HldE, we supplemented the HldE d1 and d2 domains (Fig. S1A and S4) separately in the reconstitution. The d2 domain alone in combination with GmhA only (Fig. 5C) or with both GmhA and GmhB (not shown) did not yield any active product. Using solely the d1 domain of the HldE enzyme in combination with GmhA, we obtained a proinflammatory product that we also identified as β-HMP-1 by NMR and no detectable β-D-HBP, suggesting that the HldE d1 domain activity, together with GmhA, was sufficient for HMP-1 production (Fig. 5C and 6I). The d1 and d2 domains combined behaved similar to the complete HldE enzyme in all reaction mixtures, yielding mainly ADP-heptose and a residual amount of β-HMP-1 but no detectable β-D-HBP (Fig. 6J). The alternative nucleotides

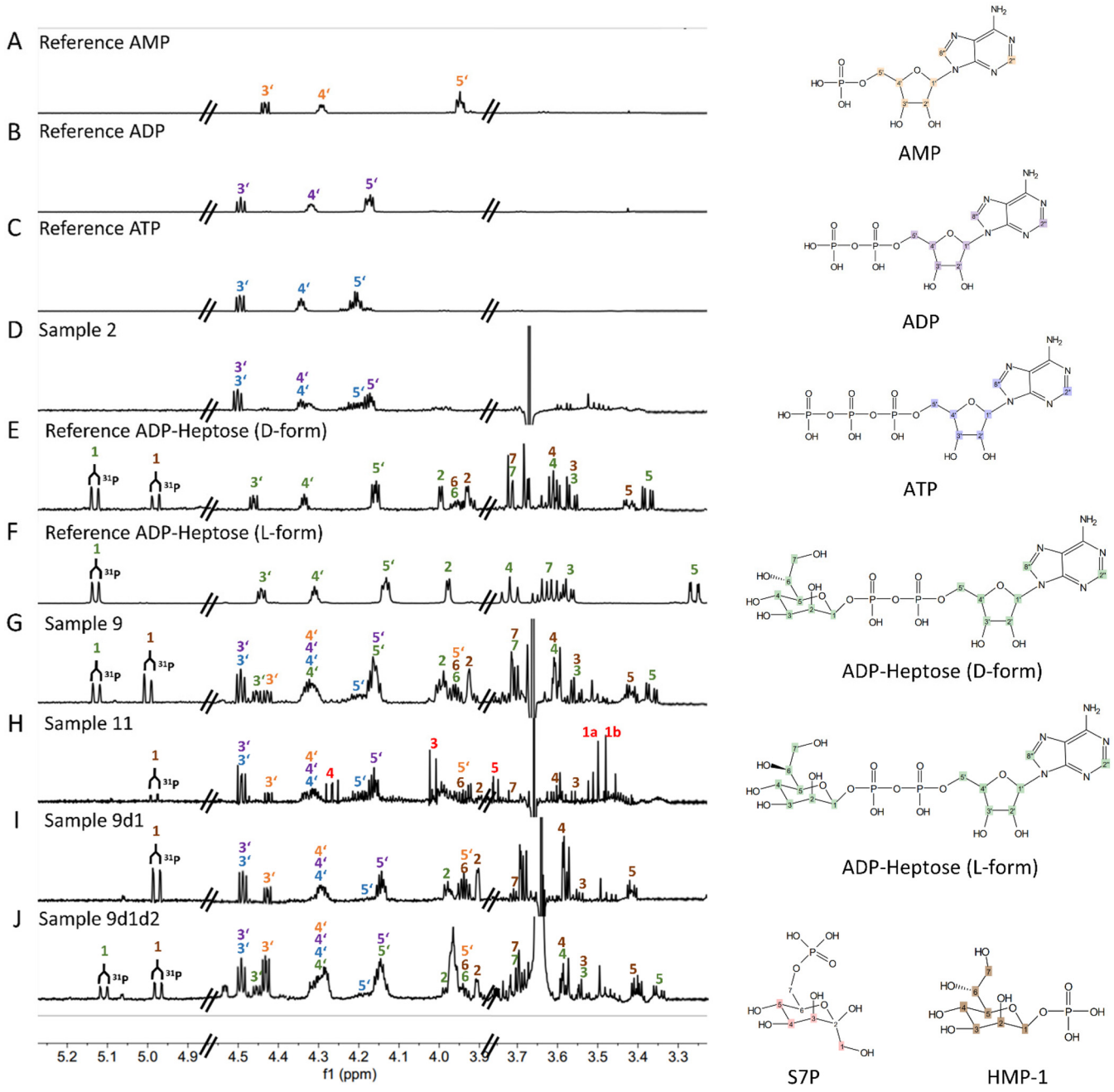

FIG 6 NMR analysis of reaction products of the *in vitro* reconstituted *H. pylori* ADP-heptose biosynthesis pathway identifies the new cell-active product β-HMP-1. *In vitro* reconstitution of the *H. pylori* ADP-heptose biosynthetic pathway was performed in one-pot reaction mixtures with various enzyme combinations as described in Fig. 5, Fig. S4 in the supplemental material, Table 3 (sample identities), and the Materials and Methods. NMR was performed as described fully in the Materials and Methods. (A to F) Control conditions and control compounds as indicated in the upper left corners analyzed in pure form (ATP, AMP, ADP, β-D-ADP-heptose, and β-L-ADP-heptose; sample 2 [D] is a control mixture without any substrate). The nomenclature of one-pot reactions (D and G to J) was as follows: sample 2, control sample with ATP and three enzymes but no substrate; sample 9, three enzymes, GmhA, HldE, GmhB, ATP, and sedoheptulose-7-P (substrate); sample 11, two enzymes, GmhA, HldE, ATP, and substrate; sample 9d1, three enzymes with HldE d1 only, ATP, and substrate; sample 9d1d2, three enzymes with HldE d1 and d2 (purified and added separately), ATP, and substrate. All enzymes used here were cloned from strain 26695a. Structures of compounds and reaction products and their respective atoms are indicated on the right; atoms are numbered and labeled in different colors. Color-coded numbered peaks in the histograms correspond to the respective atoms of the input and output compounds, shown on the right, that were detected. Detailed results for references and samples are listed in Table S3 in the supplemental material.

Na-GTP and Na-CTP, supplemented instead of ATP in the three-enzyme mixtures, did not yield detectable amounts of nucleotidyl-heptose despite pronounced proinflammatory cell activity; instead, β-HMP-1 was also revealed by NMR (not shown).

Due to the intrinsic low sensitivity of NMR spectroscopy in conjunction with severe signal overlap in the NMR spectra of crude extraction mixtures, we were not able to

detect any heptose compounds in *H. pylori* lysates by NMR. We therefore turned to mass spectrometry (liquid chromatography-electrospray ionization tandem mass spectrometry [LC-ESI MS/MS]) to test both *in vitro* reconstitution mixtures and *H. pylori* ETLs (see sample overview in Table 3) to verify compound structures and refine their detection and relative quantitation. In the *in vitro* reconstitution samples, depending on their composition, we were able to clearly detect and quantitate ADP-heptose (Fig. 7A), HBP (Fig. 7C), and the heptose-monophosphates (Fig. 7B). In these samples, the two groups of heptose-monophosphates (HMP-7/S7-P and HMP-1, respectively) can be clearly distinguished from each other by different retention times (Fig. 7B; Table 3). The largest amount of ADP-heptose product was quantitated in the three-enzyme mixture containing full-length HldE. Most HMP-1 was detected and quantitated in the three-enzyme mixtures containing either full-length HldE or HldE d1 domain only (Fig. 7B; Table 3). HBP was detected predominantly in the two-enzyme mixtures containing GmhA and HldE or GmhA and HldE d1 (Fig. 7C; Table 3).

In *H. pylori* ETLs, mass spectrometry also detected a distinct heptose monophosphate species (HMPv, probably β-HMP-1) (Fig. 7E) as a novel compound (shoulder peak in the chromatogram, matching the retention time in the reconstitution mixture containing β-HMP-1) in addition to ADP-heptose (Fig. 7D), both in the *H. pylori* wild-type strain and in ETLs of an HldE overexpression strain (Fig. 7E; Table 3). Interestingly, greatly divergent amounts of specific metabolites in two different wild-type isolates were quantitated by LC-MS, with respect to amounts of ADP-heptose and the probable HMP-1 monophosphate species (Table 3). Differences in ADP-heptose quantities between N6 and 26695a strain lysates were more than 20-fold (peak heights are in Table 3), and ADP-heptose content in lysates also varied quantitatively for a broader range of diverse wild-type clinical isolates (Fig. 7F). Similarly, proposed HMP-1 heptose-monophosphate showed quantitative differences between the two strains (Table 3). This clearly corresponded with a significant difference in proinflammatory cell activity between both strains (live and lysates) (Fig. 2). HBP, while clearly revealed in enzymatic reconstitution mixtures (Table 3), was poorly detectable by LC-MS in bacterial lysates, probably due to low concentrations and compound instability.

Medium supplementation versus cell transfection of reaction products of *in vitro* reconstitution and *H. pylori* ETLs activate cells differently: further evidence for differentially active heptose products. When we coincubated reporter cells with reaction products from the *in vitro* reconstitution reactions, we observed strong proinflammatory cell activation using the three-enzyme reconstitution (described above), mostly producing β-D-ADP-heptose according to NMR results. This activity was absent when TIFA-k/o cells were used, which cannot mount an innate immune response toward heptose metabolites (Fig. 5E), clearly assigning the activity to the heptose product(s). The cell-directed proinflammatory activity was markedly lower when coincubating reporter cells with reaction products from the two-enzyme reaction (enriched in β-HMP-1 and β-D-HBP) by merely supplementing the medium. To gather more evidence for a mixture of active products in some of the reactions, we transfected pathway reconstitution products and bacterial ETLs into the HEK-NF-κB_luc reporter cell line (Fig. 8A). While transfection of the three-enzyme pathway product (containing mostly ADP-heptose as per our biochemical analyses) elicited about the same amplitude of response as coincubating the same amount in the medium, the products of the two-enzyme reconstitution behaved differently; in this case, the transfection of the product elicited at least 3-fold higher activity in the reporter cells than medium supplementation with the same reaction products (Fig. 8A). When analyzed by mass spectrometry, the two-enzyme reaction contained markedly higher amounts of the nonpermeable metabolite β-D-HBP than the three-enzyme mixture (Fig. 8B; Table 3). We also compared the proinflammatory activity of bacterial ETLs collected from five different *H. pylori* strains (26695a, Su2, N6, L7, and Africa2) and isogenic Δ*cag*PAI or *cagY* mutants of three strains (Fig. 8C and D) on cells with and without lipofectamine transfection. In those experiments, the ETLs always activated significantly more when transfected (Fig. 8C and D). Strain differences were determined for the same amount of ETLs

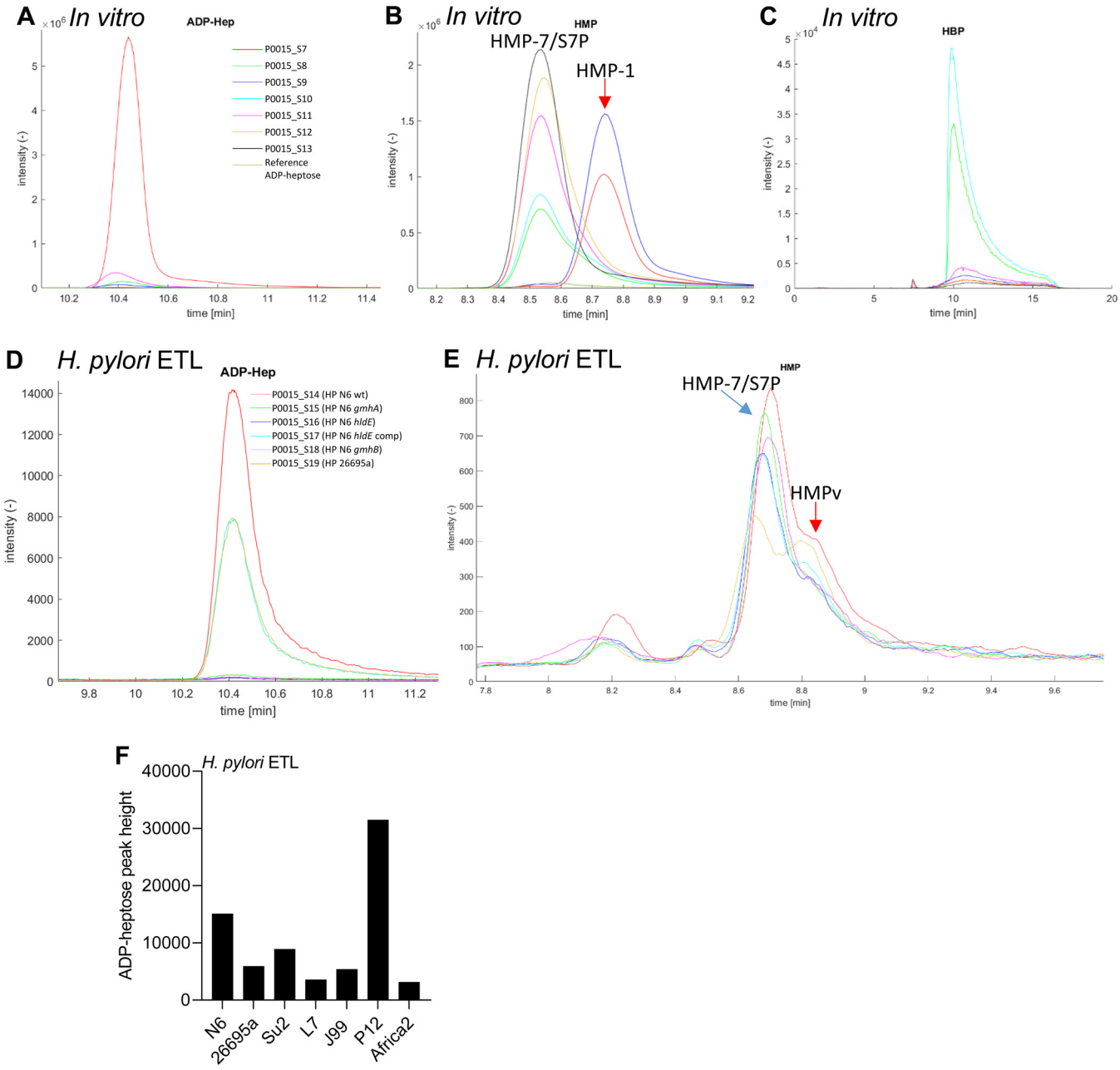

FIG 7 Mass spectrometry analysis (LC-ESI-MS-MS) confirms reaction products of the *in vitro*-reconstituted *H. pylori* ADP-heptose biosynthesis pathway and identify a new proinflammatory product β-HMP-1 and strain-specific quantities of ADP-heptose and other heptose metabolites in *H. pylori* lysates. *In vitro* reconstitution of the *H. pylori* ADP-heptose biosynthetic pathway was performed in one-pot reaction mixtures with various enzyme combinations (enzymes cloned from 26695a) as described in Fig. 5, Fig. S4 in the supplemental material, Table 3, and the Materials and Methods. Mass spectrometry was performed as described in the Materials and Methods. (A to C) Detection and quantitation of ADP-heptose (A), HMP variants (B), and HBP (C) in various one-pot mixtures of purified *H. pylori* enzymes (see Table 3). (D and E) show the detection and quantitation of ADP-heptose and HMP variants by mass spectrometry in *H. pylori* lysates (ETLs). Same color codes of chromatogram curves correspond to the same samples in A, B, and C, respectively, and in D and E, respectively. In D, samples S14 (N6 wt ETL), S17 (N6 *hldE* comp ETL) and S19 (26695a wt ETL) show strain-specific high amounts of ADP-heptose. In E, the same samples also show an increased shoulder peak compared to the other samples at the approximate retention time of heptose-monophosphate-1 (HMP-1). This shoulder is designated here as a heptose monophosphate variant, not identical to HMP-7 (HMPv), since the assignment of the shoulder peak was ambiguous. Peaks in the chromatograms are labeled with the respective compound designations (input and output compounds). See Table 3 for full descriptions of samples, compound identifications, and quantifications (peak height). (F) shows diverse ADP-heptose content (peak heights) in ETLs of seven different H. pylori wild type strains, quantitated by mass spectrometry.

using the ratio between transfected and nontransfected, quantitating how strongly the activation was increased after transfection. The ratios ranged from slightly above or below a 2-fold increase for N6 and Su2 (Fig. 8E) to between 3-fold and 4-fold for 26695a, L7, and Africa2. This corresponds to our analyses of ETLs containing (i) cell-

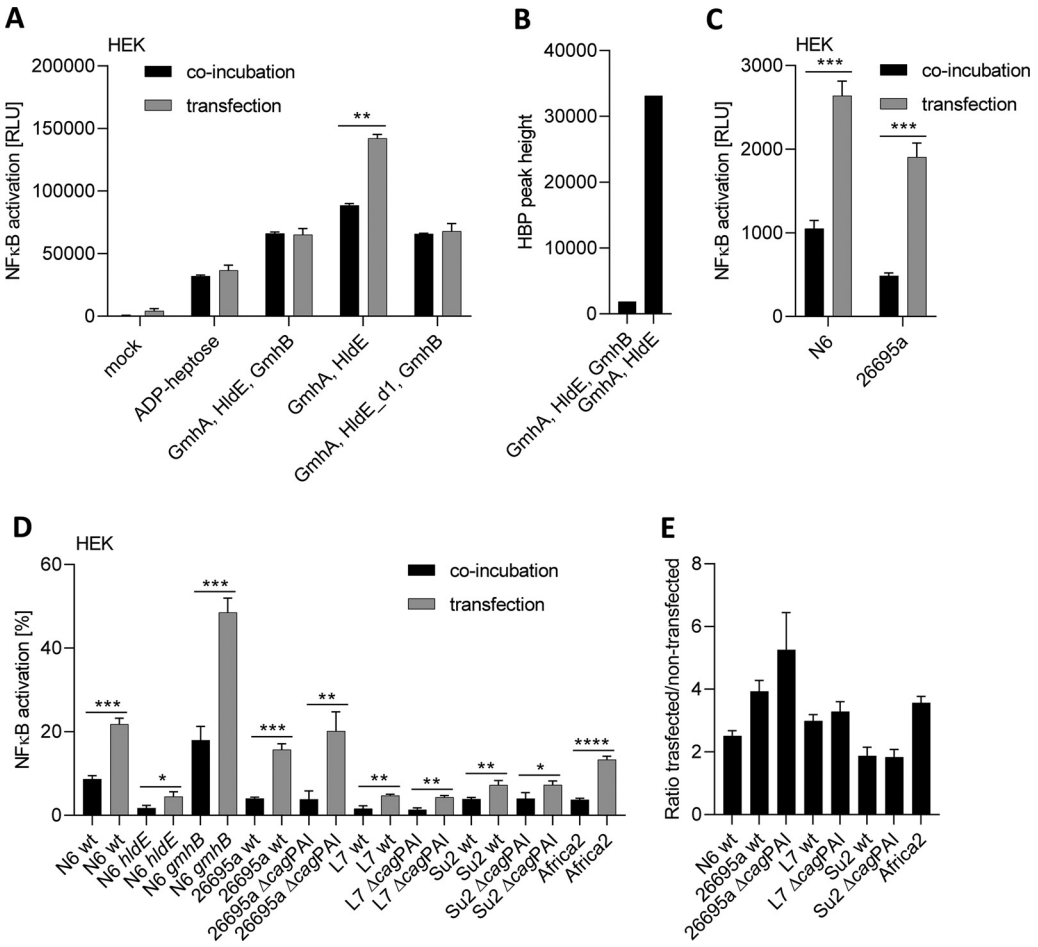

FIG 8 Transfection improves the proinflammatory activity of partially reconstituted heptose reaction (GmhA and HldE) and of some bacterial ETLs. (A) Activation of HEK-NF-κB_luc reporter cells after coincubation or transfection by reconstituted heptose mixtures produced using the recombinant *H. pylori* enzymes GmhA, HldE (complete enzyme or separate d1), and GmhB in different combinations (4 h of cell coincubation). (B) Comparative quantitation of HBP by LC-MS/MS in the reconstitution mixtures of three heptose pathway enzymes versus two enzymes (correspond to samples S7 and S8 in Table 3). The three-enzyme mixture contains very little HBP, while the two-enzyme mixture shows a more than 10-fold increase in HBP. (C) Activation potential of *H. pylori* ETLs on HEK-NF-κB_luc reporter cells after coincubation or transfection of enzymatically treated lysates (ETLs) of the *H. pylori* wild-type strains N6 and 26695a (4 h of coincubation). Reporter activation is quantitated in absolute luminescence values (RLU) in A and C. (D) Differences in proinflammatory cell activation (HEK-NF-κB_luc) between coincubation with and transfection (T) of various *H. pylori* strains' ETLs. Strain and mutant designations (wt indicates wild type of strains N6, 26695a, L7, SU2, and Africa2; mutants are designated by respective gene names) conform to previous figures. Incubation time was 4 h for all conditions; all values (shown in percent) were normalized to ADP-heptose coincubation of the same experiment (2.5 μM, not shown), which was set to 100%. Experiments to detect NF-κB activation in A, C, and D were performed in two (A) or three biological replicates each and were repeated at least once on different days. Statistically significant differences between conditions of coincubation and transfection were calculated using Student's *t* tests. Significances are marked with asterisks; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (E) Calculation of ratios between coincubated and transfected conditions for response to selected strains' ETLs (only wild-type strains and isogenic T4SS-deficient mutants, same coincubation conditions as in D).

permeable ADP-heptose (shown before in reference 8), (ii) a proinflammatory active monophosphate compound (HMPv), likely HMP-1, which also has good cell permeation activity (42), and (iii) at least one other heptose metabolite, which is less cell permeable (suggested to be HBP) (5, 8). *In vitro* one-pot reactions that cannot produce β-D-HBP (e.g., a mixture including GmhA, the d1 domain of HldE, and GmhB) and that contained HMP-1 (by NMR and LC-MS) as the only active product did not result in increased activation after cell transfection. We also tested several isogenic heptose biosynthesis and Δ*cag*PAI-mutant lysates in comparison with their wild-type parent ETLs by cell transfection (three strains). Most mutants did not behave differently from their wild-type parent (Fig. 8D and E). For strain N6, the isogenic *gmhB*-mutant ETL (expected to increase HBP product) displayed a substantial activity increase by

transfection over nontransfected conditions (Fig. 8D) and a net increase of activity over the parent strain. Interestingly, testing the 26695a *cag*PAI deletion mutant ETL revealed a stronger NF-κB activity increase by transfection over no transfection than the 26695a wild-type ETL (Fig. 8E). *cag*PAI deletion would mimic a closed T4SS. These results strongly suggest that the presence of the *cag*PAI can affect the production of metabolites that are less permeable than ADP-heptose.

DISCUSSION

Heptose metabolites from diverse bacteria have recently been characterized as MAMPs/pathogen-associated molecular patterns with strong proinflammatory activating potential for human myeloid and epithelial cells (12, 47). Some heptose metabolites, such as ADP-heptose, can be actively taken up as solutes after they have been released into the environment (5, 7, 8, 42). A number of pathogenic bacteria, including *H. pylori* and some *Enterobacteriaceae*, do not release a substantial amount of heptose metabolites into the medium but use their host-directed secretion systems (T4SS and T3SS) for direct transfer of the metabolites into host cells (4, 8, 11, 12, 14). This mode of targeted metabolite transport raises the obvious questions of whether and how bacteria use active mechanisms to regulate the activating metabolite biosynthesis, possibly in a strain- and cell contact-specific manner. Envisaged mechanisms would comprise specific sensing of environmental cues and subsequently lead to increased activation of heptose biosynthesis genes after host cell contact.

Using the model organism *H. pylori*, which translocates ADP-heptose and possibly other heptose metabolites via its type 4 secretion system, we have addressed several questions regarding strain diversity in heptose gene regulation, heptose metabolite generation, and variable proinflammatory effects of bacterial heptoses on host cells. First, we obtained a fundamental knowledge on strain diversity in transcript amounts and transcriptional and posttranscriptional regulation mechanisms of the *H. pylori* heptose biosynthesis gene cluster. Transcript amounts varied between strains and between the genes of the cluster in each tested strain, with the last genes of the cluster, and the most relevant genes for generating proinflammatory heptose intermediate products (11), *gmhA* and/or *hldE*, frequently possessing the highest transcript activities. Both the absolute transcript amounts of each gene and relative expression patterns of the cluster genes were extraordinarily strain variable. In particular, we found strains with very high transcript amounts specifically of *gmhA* and *hldE*, while other strains, such as L7 (Asian origin) and Africa2 (a primary *cag*PAI-negative strain), had higher transcript amounts selectively of both *gmhA* and *gmhB* but not of *hldE*. These observed expression differences can explain some of the strain differences in proinflammatory bacterial lysate activity and in cell responses induced by live bacteria that have been observed in this work and before (41). In addition, while *gmhA*, *gmhB*, and *hldD* did not differ markedly in sequence between *H. pylori* strains, the bifunctional *hldE* gene, crucial for the generation of proinflammatory cell-active heptose metabolites (3, 8, 10, 11), showed a comparably strong interstrain DNA and derived amino acid sequence variability. Interestingly, using a specific antiserum against HldE, we could also pin down marked strain-specific differences in HldE protein content. The finding that the different strains had a very diverse heptose-dependent proinflammatory activation potential on epithelial cells as shown here and previously (11, 41) is an expected outcome of expression differences and HldE sequence diversity. HldE protein expression in different mutants (*cagY* and Δ*cag*PAI) of the same strain appeared not much changed except for the HldE mutant. Taken together, our findings of transcript profile diversity and strain-variable HldE sequence and protein content suggest a major role of this bifunctional enzyme in host interaction. Since bacterial lysates do not permit the detection of metabolite transport differences of live bacteria, we still have less insight into active metabolite production and content in live bacteria in the presence of target cells. Upregulation of the heptose cluster genes in wild-type bacteria with an active T4SS in the presence of cells is highly suggestive of increased heptose metabolite biosynthesis after cell contact.

To identify novel proinflammatory heptose metabolites that *H. pylori* can variably produce, and to identify strain-specific differences in enzyme activities as one possible

explanation for interstrain variation of active metabolite content, we reconstituted the heptose biosynthesis pathway in one-pot reaction mixtures. We used purified HldE enzyme from two different *H. pylori* strains (26685 or N6) and two separate HldE domains. When we tested the metabolic reaction products on NF-κB reporter cells, the fully reconstituted pathway mixture (the three enzymes GmhA, HldE, and GmhB combined) as well as the combination of GmhA and HldE (two enzymes only) both produced proinflammatory cell-permeable active metabolites, although the latter reaction cannot form ADP-heptose. Transfection of reporter cells with pathway reconstitution products revealed that the two-enzyme reaction products acted much stronger after transfection, while the three-enzyme product (predominantly β-D-ADP-heptose and HMP-1 according to NMR and mass spectrometry analyses) showed no difference between transfection and supplementation in the cell medium. The *H. pylori* pathway had been only partially enzymatically reconstituted *in vitro* before the present study, using synthetic β-HMP-7 or β-HMP-1 as substrates and GmhB and/or HldE as purified enzymes (8). In the latter study, only β-D-ADP-heptose was identified as the reaction end product in *H. pylori* lysates (using mass spectrometry), which is cell permeable, while β-D-HBP, an intermediate pathway product, is not (5). The reconstituted heptose pathway from *Campylobacter jejuni*, in a three-enzyme mixture, was previously assessed to activate cells by external supplementation (7), while the latter study did not characterize the reaction products further. We established differences in the processive enzyme activities of HldE proteins from two different strains (26695a and N6), which show considerable amino acid sequence diversity, using the one-pot mixtures followed by LC-MS analysis of the products. HldE of strain N6, alternatively added to the enzyme mixtures, was significantly more active, even as a single enzyme or in the two-enzyme combination with GmhA and produced significantly more ADP-heptose detectable by mass spectrometry, both in the enzyme mixtures and in *H. pylori* lysates (Fig. 7D). The findings of interstrain transcript, regulatory, and enzyme variation in the heptose biosynthesis pathway points to a variety of factors that can influence strain diversity in cell-directed metabolites and proinflammatory activity.

In the present study, we could now also unequivocally assign major reaction products formed in one-pot reaction mixtures containing recombinant *H. pylori* enzymes, and in *H. pylori* lysates, using both NMR spectroscopy and mass spectrometry. In the two-enzyme mixtures, we identified β-D-HBP by mass spectrometry. By NMR, this reaction mixture also identified a second proinflammatory metabolite heptose-monophosphate variant β-HMP-1, which had been synthesized *in vitro* as a novel cell-active heptose product previously (42) but had not yet been identified in bacteria directly. β-HMP-1 can theoretically be formed already in the second enzymatic step of the biosynthesis reaction, which we could confirm by NMR in a mixture using only the HP0858 d1 domain in addition to GmhA. By mass spectrometry, HMP-1 was not identified in the two-enzyme mixtures, which is probably due to detection limits inherent in the methodology. Both by NMR and mass spectrometry, HMP-1 and β-D-ADP-heptose were readily detectable in the three-enzyme mixtures. In *H. pylori* lysates, depending on strain and mutant identity, we quantitated as main cell-active products both ADP-heptose and a probable monophosphate (likely HMP-1), which has never been identified directly in bacteria. Particularly intriguing were the strong differences in amounts of ADP-heptose and proinflammatory heptose-monophosphate quantities between tested wild-type strains. From transfection experiments of lysates and the two-enzyme preparations, we assume that, additionally, a third active compound other than ADP-heptose and probable HMP-1 is contained. HBP, which was only detectable in one tested strain (N6) by mass spectrometry, has been detected before in low quantities and was found to not be cell permeable (4, 8, 42). We established that the activities and metabolite content of wild-type lysates and *in vitro* reaction mixtures containing HldE proteins from two different *H. pylori* isolates (N6 and 26695), which show considerable amino acid sequence diversity, were different. HldE reaction mixtures of strain N6 were significantly more enzymatically active, even as a single enzyme or in the two-enzyme combination with GmhA, to produce HMP-1 or ADP-heptose. This matched to the higher

detectable content of metabolites in strain N6 bacterial lysates, which was only exceeded by another frequently used strain P12 (8). Strain differences in the production of β-D-ADP-heptose and other heptose metabolites by *H. pylori* can therefore be detected and partially quantitated directly in lysates. Strain differences in heptose metabolite production can also be inferred in our present study from (i) distinct transcript amounts of various heptose cluster genes between different strains, (ii) varying enzyme activities, or (iii) the strain-variable ratios between lysate-transfected and coincubated conditions. In any case, for the production of cell-active intermediate pathway metabolites, the central bifunctional enzyme HldE is essential. Through our *in vitro* reconstitution, we also collected evidence that domain d1 of HldE might, strain-specifically (e.g., in N6), act not only as a kinase but also as a phosphatase *in vitro*. This finding may resolve the conundrum that in distinct live *H. pylori* isolates, the phenotype of heptose metabolite-dependent cell activation of HP0860 (*gmhB*) mutants was found to be strain-dependently distinct (8, 11). Since we demonstrated that the *H. pylori* heptose biosynthesis pathway can produce a proinflammatory intermediate metabolite HMP-1 in the first two steps already with only GmhA and HldE, the next enzymatic steps may, in principle, not be necessary to produce proinflammatory metabolites.

Expressed separately, the two domains of the HldE enzyme, corresponding to the different enzymatic domains, had differential effects when used as part of the reconstitution reaction; single d2 addition in the three-enzyme mixture did not lead to any cell-active product, while single d1 addition also produced detectable cell-active β-HMP-1 (NMR and mass spectrometry). Activity of the latter reaction product was not higher when transfected into cells, different from the products of the GmhA and HldE two-enzyme reconstitution, supporting again the notion that a nonpermeable product (HBP) causes the additional activation. The use of different nucleotides as cofactors in the reaction revealed that nucleotides are essential cofactors for all steps of the reactions to proceed. Without nucleotides, no products are revealed. Second, nucleotides other than ATP (CTP and GTP) can serve as cofactors of the enzymes in the first two reaction steps as efficiently as ATP but cannot be transferred into nucleotidyl-heptose by HldE, as the reaction product then stopped at β-HMP-1. Adekoya et al. (42) demonstrated that, when provided at equal molarities with transfection, β-HMP-1 was about equally active on HEK epithelial cells as β-D-HBP, while HMP-7, the primary intermediate reaction product generated from S7-P by GmhA (also detected in some of our samples), was not cell active at all. Our coincubation results also confirmed that various human cell types can take up ADP-heptose and β-HMP-1 metabolites from the medium without the need of a bacterial transport system (12).

In addition to strain-specific traits in transcript amounts and enzyme and lysate activities of the heptose biosynthesis gene cluster, we identified a role of the presence and activity of the *cag*PAI and the CagT4SS. In mutants not possessing the *cag*PAI, the heptose cluster genes were expressed less than in the corresponding wild-type strains. A closed or absent secretion system, inducing feedback regulation, broadly influenced bacterial gene regulation in different functional categories, emphasizing metabolic and motility functions. The presence of cells also modulated bacterial gene regulation and, in turn, upregulated heptose pathway transcripts. Our regulation analyses therefore suggest that heptose cluster transcript activities and pathway output can be adjusted according to T4SS activity and the presence of cells. Prompted by the pathway analysis of the comprehensive differential transcriptomes in *cag*PAI-deleted bacteria, we obtained evidence that the modulation of the heptose gene cluster in *H. pylori* is at least partially under CsrA regulation (43, 48–50). CsrA is an important metabolic and global posttranscriptional bacterial regulator acting on RNA, which broadly influences metabolic pathways and motility functions in various bacteria, including *H. pylori* (46, 48–51). A preliminary definition of a genome-wide set of CsrA-dependent transcripts in *H. pylori* was performed previously (43), and our comprehensive data set of T4SS-dependent regulation in the present study showed some overlap. Recent work in *E. coli* has impressively demonstrated how CsrA acts on a genome-wide scale both as an activator and a repressor (46). We generated and characterized *H. pylori csrA* mutants, which demonstrated that the heptose gene cluster, in addition to known CsrA-regulated

transcripts, is under CsrA control and that *csrA* mutants also exert lower proinflammatory activity on human cells. As one signature gene downstream of CsrA, whose activity is governed by fluxes in carbohydrate metabolism (52), we also confirmed *fbp* (encoding fructose-bisphosphatase). The Fbp reaction product, fructose-1,6-bisphosphate, is an upstream modulator of central metabolism and glycolytic flux (glycolysis and tricarboxylic acid [TCA] cycle activation versus gluconeogenesis) in bacteria (53). We found increased *fbp* transcript expression alongside *csrA* after bacterial coincubation with human gastric epithelial cells. The results are suggestive of a global regulatory switch of *H. pylori* phenotype between ecological conditions (e.g., planktonic versus cell-associated) under which cell-directed heptose transport and other functions are either shut off or switched on.

In conclusion, *H. pylori* produces a proinflammatory heptose-monophosphate MAMP, most likely β-HMP-1, in addition to β-D-ADP-heptose and HBP. Additionally, heptose biosynthesis pathway activity and HldE expression are regulated strain specifically and are dependent on CagT4SS activity, which entails partial CsrA-dependent regulatory feedback on heptose biosynthesis and global functions. Hence, intracellular heptose metabolite amounts and an active CagT4SS are likely to influence bacterial central metabolism and induce cell contact-dependent heptose production "upon" demand. Consequently, *H. pylori*, an important model pathogen and paradigm of persistently host-associated bacteria, increases its toolbox of mechanisms to influence and fine-tune its production of cell-active, translocated, proinflammatory carbohydrate metabolites and thereby possibly combines innate immune activation of host cells with metabolic cross-talk.

MATERIALS AND METHODS

Bacterial strains and culture conditions of *H. pylori* strains. *H. pylori* bacteria of various wild-type strains and corresponding isogenic allelic exchange mutants in heptose biosynthesis genes, *cagY*, and the *cag*PAI were used as listed with references in Table 1. The mutants were generated as reported previously (11). For novel *cag*PAI deletion mutants in L7 and Su2, the insertion mutations were recreated by the same strategy as previously described (11). The *cag*PAI deletion mutant is a complete deletion, while all single-gene mutants were designed as insertion mutations to retain 3′ and 5′ segments of the genes interrupted by a kanamycin resistance cassette. For routine culture, *H. pylori* strains were grown on blood agar plates (Oxoid blood agar plates base II) supplemented with 10% horse blood (Oxoid) and the following antibiotics for selective growth of insertion mutants (all purchased from Sigma-Aldrich, USA): amphotericin B (4 mg/L), polymyxin B (2,500 U/L), vancomycin (10 mg/L), trimethoprim (5 mg/L), and kanamycin (optional; 10 mg/L). For cell coincubations, transcriptome analyses, and preparations of lysates, bacteria were freshly grown for 20 to 24 h on plates in anaerobic jars supplemented with Anaerocult C sachets (Merck, USA) at 37°C. A list of strains used in the present study is provided in Table 1.

***H. pylori csrA* mutant.** The *H. pylori csrA* mutant was generated by inserting a kanamycin cassette (aphA3′-III) into the *H. pylori csrA* gene and cloning it into pUC18 using PCR and *csrA*-specific primers (Table 4). The construction of the *csrA* mutant was performed with initial amplification of *csrA* (HP1442) and *csrA*-flanking regions from *H. pylori* 26695 genomic DNA and introducing PstI and KpnI restriction sites (resulting in plasmid pCJ2008). Primers HP_1442del_bglII_fw and HP_1442del_bglII_rv (Table 4) were then used to reverse amplify and religate plasmid pCJ2009 from pCJ2008. pCJ2009 contains the restriction site BglII in the center of *csrA*, with a 42-bp partial deletion of the gene. Using the BglII site, the kanamycin cassette (cut BamHI) was inserted, generating plasmid pCJ2010. The resistance cassette insertion in the chromosomal *csrA* locus of *H. pylori* strain N6 was generated after natural transformation of pCJ2010 by allelic exchange mutagenesis. *csrA* insertion mutants were selected on blood agar containing kanamycin and checked for correct chromosomal insertion-recombination using cloning primers HP_1442_PstI_fw and HP_1442_KpnI_rv and resistance cassette-specific primers.

Cultivation of human cells. The human cell lines AGS (ATCC, CRL-1739; human gastric adenocarcinoma cell line) and MKN28 (JCRB0253; human gastric carcinoma cell line) were routinely cultured in RPMI 1640 medium buffered with 20 mM HEPES and GlutaMAX stable amino acids (Gibco, Thermo Fisher Scientific, USA). Medium was supplemented with 10% fetal calf serum (FCS; PromoCell, Germany). AGS and MKN28 cells were cultured without antibiotics. The cell line HEK-NF-κB_luc (BPS Bioscience, USA; luciferase reporter cell line) was routinely cultured in Dulbecco's modified Eagle's medium (DMEM; buffered with 20 mM HEPES) supplemented with GlutaMAX (Gibco, Thermo Fisher Scientific, USA) and 10% FCS (PromoCell, Germany). HEK-NF-κB_luc cells were supplemented for routine growth with 50 μg/mL hygromycin B (Invivogen, USA) in the same culture medium. For infection and coincubation experiments, all cell lines were seeded in medium without antibiotics. Antibiotics were removed from all cell cultures by washing before starting coincubation assays. All cell cultures were grown in a 5% CO_2 atmosphere incubator and routinely passaged using 0.05% buffered trypsin-EDTA (Gibco, Thermo Fisher Scientific, USA).

Coculture of cells with live bacteria or bacterial products. Human cells were cocultured with various *H. pylori* strains or bacterial lysates. The infection was performed in 6-, 24-, or 96-well plates (Greiner Bio-One, Austria) on subconfluent cell layers (60 to 80% confluence) seeded on the previous day. Sixty minutes before infection, the medium was exchanged to fresh RPMI 1640 (supplemented with 20 mM

TABLE 4 Primers for cloning

Primer name	Sequence (5′→3′)	Description
HP0858xp_BamHI_fw	TATGGATCCAAAAAAATCTTAGTCATAGGCGATCTGA	Cloning of HP0858 d1 of strain HP26695 in pET28a (His tag in frame); cloning of HP0858 (complete gene) of strain HPN6 in pET28a (His tag in frame)
HP0858_rv5	TATGCGGCCGCTCATTCTAAAGTTTCTAACAGCTTTTCTAAAG	Cloning of HP0858 d1 of strain HP26695 in pET28a (His tag in frame)
HP0858_fw5	TATGGATCCCAAAAAATCGTTTTCACCAATGG	Cloning of HP0858 d2 of strain HP26695 in pET28a (His tag in frame)
HP0858xp_NotI_rv	TATGCGGCCGCTCAATCATTGCATGTCCTTTTAATTTTTTCTA	Cloning of HP0858 d2 of strain HP26695 in pET28a (His tag in frame); cloning of HP0858 (complete gene) of strain HPN6 in pET28a (His tag in frame)
HP1442del_PstI_fw	AAACTGCAGATTATCCAACTTACCGCTTA	Cloning of HP1442 (*csrA*) and flanking regions into pUC18
HP1442del_KpnI_rv	AAAGGTACCGCCATAAGAAGTGGCGTTAG	Cloning of HP1442 (*csrA*) and flanking regions into pUC18
HP1442del_BglII_rv	AAAAGATCTCTTTGCGGCTGAGTATGAGC	Reverse amplification and insertion of restriction site for *aphA3′-III* (km)[a] cassette
HP1442del_BglII_fw	AAAAGATCTAGAGGGAGTGTGCGTTTAGG	Reverse amplification and insertion of restriction site for *aphA3′-III* (km)[a] cassette

[a]km, kanamycin; restriction sites are underlined.

HEPES, GlutaMAX, and 10% FCS) or fresh DMEM. All bacteria-cell or compound- and lysate-cell coincubation experiments were performed in the absence of antibiotics. For infection of cells with bacteria, *H. pylori* was harvested after 20 h of growth from fresh blood plates into cell culture medium. The optical density at 600 nm (OD_{600}) of this bacterial suspension was measured and adjusted to the respective multiplicities of infection (MOIs; an MOI of 25 was used in most experiments), as indicated in the results and figures. The coincubation was synchronized by centrifugation of the cell culture plates (300 rpm for 5 min at room temperature). For coincubation with enzymatically treated lysates (ETLs) or β-D-ADP-heptose (Invivogen), cell medium was also changed 60 min before the addition of lysate preparation or β-D-ADP-heptose to the cells to fresh medium without antibiotics, and coincubation started with centrifugation of the plate. Coincubation was performed in a 5% CO_2 atmosphere cell incubator for different incubation periods (indicated in text and figures). Samples were harvested after taking off the supernatant carefully (for ELISA, IL-8 secretion), either by scraping the cells from the bottom of the plate (for RNA isolation) or by adding luminescence substrate to the cells and medium (for NF-κB luciferase quantitation). If not otherwise indicated, cells were coincubated with bacteria or metabolite preparations for 4 h for measuring IL-8 secretion or for performing luminescence reporter assays.

RNA isolation of human and bacterial samples. RNA was isolated from human cells, bacteria, or bacteria-cell coincubation samples. Human cells (cocultured) were harvested from 6-well plates (Greiner Bio-One, Austria) or small petri dishes (diameter of 3 cm) by scraping the cells from the surface using a rubber policeman. *H. pylori* strains were harvested by resuspending bacteria from plates after approximately 20 h of growth. Human coincubated or bacterial samples were centrifuged, and pellets were snap-frozen immediately in liquid nitrogen or on dry ice and stored at −80°C until RNA preparation. From pellets, total RNA was isolated using an RNeasy minikit (Qiagen, Germany) following the manufacturer's instructions after mechanical lysis of the samples in a FastPrep bead beater (MP Biomedicals, Inc., USA) at 5 MHz for 45 s using lysing matrix B (for bacterial samples; MP Biomedicals). Isolated RNA was treated with DNase using a TURBO DNase cleanup kit (Ambion-Invitrogen, USA). Sufficient RNA quality and purity were ensured by photometric measurement, gel electrophoresis, and RNA ScreenTape analysis in a Tape Station (Agilent, USA) using high-sensitivity RNA tapes and by the amplification of control genes (*H. pylori* 16S rRNA genes for control of RNA purity). Total RNA was then used for genome-wide RNA-sequencing (RNA-seq) or further processed for cDNA generation and performance of RT-qPCR.

cDNA synthesis and RT-qPCR. cDNA was synthesized from 1 μg of total bacterial RNA using Superscript III reverse transcriptase (Invitrogen, USA), RNase-Out (Invitrogen, USA), and random nonamer primers. Reverse transcription was performed from 1 μg of total RNA. All reagents were purchased from Invitrogen (USA). Sufficient quality of cDNA was ensured by control PCRs (amplification of 16S for bacterial cDNA).

RT-qPCR was routinely performed on 0.5 μL of cDNA in a CFX96 real-time PCR cycling machine (Bio-Rad, USA) using *H. pylori* gene-specific primers (synthesized at Metabion) (Table 5) and 2× SYBR Green master mix (Qiagen, Hilden, Germany). Transcript quantification was always performed in triplicate, with gene-specific standards for absolute amount quantification, and performed in parallel for each transcript using the following protocol: 95°C for 10 min; 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles; and a melting curve of 60°C to 95°C with an increment of 0.5°C for 5 s. Results were equalized to 1 μL of cDNA and normalized to transcript amounts of the *H. pylori* 16S gene using a correction factor respective to the mock reference but maintaining the absolute values (pg/μL) for all final transcript amounts. MiQE standards for the RT-qPCR method were applied as detailed in reference 37. Statistics for Fig. 1C to F are provided in Table S1 in the supplemental material. Each figure shows different biological experiments, which explains the slight variation of absolute transcript quantities. Different experiments can have slightly different starting quantities/transcript amounts of certain transcripts due to biological variation.

TABLE 5 List of gene-specific primers used for RT-qPCR

Primer name	Sequence (5′→3′)	Description
HP0857_qPCR_fw1	TAGCCCATAAGGAAGCGTTA	Amplification of gene HP0857
HP0857_qPCR_rv1	GTCAATTCAGCGGCAAAATG	Amplification of gene HP0857
HP0859_qPCR_fw1	GCATTTTGATTATTTGTTCCACC	Amplification of gene HP0859
HP0859_qPCR_rv1	CGCTGAAGAAGCGTAAATCA	Amplification of gene HP0859
HP0860_qPCR_fw1	CAGAGACGGCATTATCAATATTG	Amplification of gene HP0860
HP0860_qPCR_rv1	GATTGGTTGGTGATTAAAAGCA	Amplification of gene HP0860
HP0858_qPCR_fw1	CGAGTTTAGAAGAAATCGCT	Amplification of gene HP0858
HP0858_qPCR_rv1	CCTAAAGCTTTAGCCTTTTGC	Amplification of gene HP0858
HP1385_qPCR_fw1	TAAAGCGGATTTAGCCCTAG	Amplification of gene HP1385
HP1385_qPCR_rv1	CATAAGCGATCAAATAAGAGCC	Amplification of gene HP1385
HP1442_qPCR_fw1	GAAGGGATTGTCATTGATGATAAC	Amplification of gene HP1442
HP1442_qPCR_rv1	ACAATGGCCTCTTTGAGTTC	Amplification of gene HP1442

RNA-seq and data analysis. RNA-seq and transcriptome analyses were performed as previously described (12). Briefly, paired, trimmed, and quality-filtered fastq files with an average read length of 150 bp obtained from the Illumina NextSeq2500 platform were processed using the CLC Genomics Workbench version 20.0 (Qiagen, Germany). Paired reads were downsampled for each sample to 2,000,000 and mapped against the *H. pylori* reference genome 26695 (accession number AE000511, NCBI database) (53). Reads were aligned as previously described (12), and gene expression levels were quantitated as reads per kilobase per million (RPKM) values normalized to the gene length and read number. RPKM values were then used for calculating differential expression between samples using the CLC Genomic Workbench RNA-Seq analysis workflow. See Table S2 for full transcriptome results and differentially expressed genes. Full results are accessible on NCBI's Gene Expression Omnibus (GEO) under accession number GSE227450.

ETLs and water lysates. Bacterial lysates were prepared from *H. pylori* harvested into sterile cell culture 1× phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific, USA) from blood agar plates after 20 h of growth. The bacterial suspension, quantified by OD_{600} measurement, was centrifuged, and the pellets were stored at −20°C. Briefly, for lysate preparation, the frozen pellet was resuspended in an appropriate volume (1 mL) of 1× PBS and adjusted to an OD_{600} of 2 (per mL). The suspension was boiled for 10 min in a water bath (99°C) and centrifuged. The supernatant was sterile filtered (0.22-μm-pore filter; Merck Millipore, Germany) and stored at −20°C if not further processed immediately. Further processing of the samples was performed either by enzymatic treatment (generation of ultrapure ETLs), as described previously (11), or by methanol precipitation. For NMR measurements (less-pure lysate preparations, which still contain, for instance, residual DNA), lysates of an OD_{600} of 20, 10, or 5 in 1 mL were prepared in PBS as described above and boiled for 20 min. These lysates were then additionally treated for protein precipitation with 3 volumes of ultrapure methanol (Sigma-Aldrich, USA). Ultimately, samples were vortexed and centrifuged to precipitate proteins and to recover protein-free supernatants, which were again sterile filtered. Ultrapure ETL samples used for mass spectrometry were additionally mixed with methanol and acetonitrile (1:2:2 [vol/vol/vol] final mixture).

Cytokine measurement from cell supernatants. IL-8 secretion from human cells to the supernatant was quantified by performing a human IL-8 ELISA, according to the manufacturer's protocol (BD OptEIA, 555244), on pretested sample dilutions. Colorimetric signal detection was performed using a Clariostar multiwell plate-reading machine (BMG Labtech).

Luciferase quantitation in human reporter cells. Briefly, firefly luciferase signal of HEK-NF-κB_luc cells was determined using a Steady Glo/Bright Glo luciferase assay (Promega, USA), as previously described (12). Luciferase quantification was regularly performed in 96-well F-bottom plates (Greiner BioOne, Austria, 655180) using 50 μL of total sample volume. After 4 h of coincubation with live bacteria, bacterial lysates, β-D-ADP-heptose, or other metabolites (as indicated in the Results and figures), equal volumes of the luciferase lysing and detection buffer (Promega) were added to each well. The reaction was allowed to incubate for 10 min for cell lysis with shaking, followed by luminescence measurement in a Victor Nivo Multimode microplate reader (PerkinElmer, USA) at the following settings: shaking for 3 s, no filter, 1-s photon counting. All conditions were analyzed in duplicate or triplicate.

SDS-PAGE and Western blotting for protein detection and quantification. Bacterial samples were prepared by harvesting bacteria directly from the plate, after centrifugation, into 1× PBS, followed by ultrasonication (Branson sonifier, 2 × 1 min at power setting 5) and subsequent separation of soluble and insoluble fractions by centrifugation (10,000 × *g*, 20 min, 4°C). The concentration of protein in each sample was determined by bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, USA) using a Clariostar multiwell reader for final colorimetric readings. Regularly, 10 μg of protein (equalized amounts for all samples) was loaded onto 11.8 to 14% SDS gels and run at 100 V constant voltage in Laemmli buffer supplemented with 0.1% SDS. Blotting was performed onto BA85 nitrocellulose membranes (Schleicher & Schuell) in Towbin buffer for 2 h at 300 mA. Blotted membranes were blocked using 5% skim milk (Sigma-Aldrich, USA or Bio-Rad, USA) or 1 to 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer containing 0.1% Tween (TBS-T), according to antibody specifications. Specific antibody (Table 6) incubations were performed for 1 h at ambient temperature or overnight at 4°C. As secondary antibody, goat anti-rabbit or goat anti-mouse antibody coupled to horseradish peroxidase (HRP; Jackson Immuno Laboratories) was used at a dilution of 1:10,000. Signal was detected using Immobilon

TABLE 6 Specific antisera and antibodies used in this study

Antigen	Source species	Reference
HldE	Rabbit	This study
FlhA	Rabbit	61

HRP chemiluminescence substrate detection reagent (Merck Millipore, Germany) and imaged in a chemiluminescent imager (Bio-Rad, USA).

DNA cloning methods for protein expression. For expression of heptose biosynthesis enzymes, genes HP0857 (*gmhA*), HP0858 (*hldE*), and HP0860 (*gmhB*) were cloned from PCR products generated from genomic DNA of the respective *H. pylori* wild-type strains, 26695a and N6, using BamHI and NotI (New England BioLabs [NEB], USA) as full-length constructs in pET28a(+) (EMD Biosciences, Novagen, Germany). The two strains were selected for their diversity in *hldE* sequence. To express the two domains of the bifunctional enzyme HldE (HP0858), the first (amino acids 1 to 325) and last segments of the gene (amino acids 328 to 461) were cloned separately into pET28a(+) using the same restriction enzymes. Gene constructs were located behind a 6× N-terminal His tag, separable from the enzyme using the tobacco etch virus (TEV) protease cleavage site between construct and His tag. The plasmids confer kanamycin resistance allowing selection of clones. Clones were checked by restriction analysis and Sanger sequencing of the complete inserts. The following were the resulting plasmids: HP0858/*hldE* (strain 26695) is pCJ1628, HP0858/*hldE* (strain N6) is pCJ2004, HP0857/*gmhA* (26695) is pCJ1627, HP0860/*gmhB* (26695) is pCJ1630, HP0858/*hldE* (26695) d1 is pCJ2002, and HP0858/*hldE* d2 is pCJ2003. The primers used for cloning are listed in Table 4.

HEK cell transfection for metabolite activity testing (ETLs). HEK-NF-κB_luc reporter cells (BPS Bioscience, USA) were transfected with bacterial ETLs or *in vitro*-synthesized heptose metabolites using Lipofectamine 2000 transfection agent (Invitrogen). Transfection was performed in 96-well plates, and cells were seeded to 3×10^4 cells in 50 μL of DMEM (containing 10% FCS) per well approximately 20 h before transfection. Medium was exchanged to 25 μL of Opti-MEM (containing 5% FCS) 1 h before the addition of 25 μL of transfection agent containing 4 μL of ETL or heptose metabolites, 0.5 μL of Lipofectamine 2000, and 20.5 μL of Opti-MEM per well. Transfected cells were subsequently incubated at standard culture conditions; after 4 h of incubation, luciferase substrate buffer (SteadyGlo, Promega) was added for cell lysis, and luminescence was detected as described above.

Protein expression and purification from *E. coli*. Expression of heptose biosynthesis enzymes from inducible expression plasmids was performed in Luria-Bertani (LB) broth (Lennox [Oxoid]) supplemented with 50 μg/μL kanamycin (Sigma, USA) from expression cultures set up in *E. coli* Rosetta pLysS (Novagen/Merck, Germany) inoculated from overnight culture to a starting OD_{600} of 0.1. At an OD_{600} of 0.5 to 0.8, expression cultures were induced with 0.1 to 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown to express protein for 4.5 h at 30°C (with 175 rpm shaking). Bacterial pellets were harvested by centrifugation (10,000 × *g*, 10 min, 4°C) and stored at −20°C before further purification. Proteins were purified from the soluble (native) fraction after the lysis of bacterial pellets by ultrasonication using a 1-mL nickel-nitrilotriacetic acid (Ni-NTA) fast protein liquid chromatography (FPLC) affinity chromatography column (Macherey & Nagel, Germany) coupled to an Äkta Purifier or Prime system (Cytiva/GE Healthcare, USA) at 20°C (room temperature) or 4°C (the latter for N6 HldE and 26695 GmhB). After loading the column, nonspecific impurities were removed by extensively washing the column with purification buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, and 1 mM dithiothreitol [DTT]) and high-salt purification buffer (1 M NaCl added to the previous), followed by equilibration in purification buffer. Gradient elution was performed by increasing the imidazole concentration of the purification buffer/lysis buffer gradually from 0 to 500 mM. High-protein-content elution fractions were pooled, dialyzed into buffer without imidazole, and further characterized, and protein purity and quantity were analyzed in SDS gels using appropriate reference protein loadings.

Generation of an anti-HldE antiserum. The antiserum was raised in rabbits against overexpressed, column-purified, full-length *H. pylori* HldE protein (cloned from strain 26695).

***In vitro* reconstitution of the *H. pylori* heptose biosynthesis pathway.** All three central enzymes of the pathway (GmhA, HldE, and GmhB), cloned from *H. pylori* into the pET28a vector, were overexpressed and purified from *E. coli* by Ni^{2+}-affinity chromatography (see earlier). All proteins were purified from the soluble fraction in native form. *In vitro* reconstitution of the biosynthetic pathway was performed as follows: about 5 to 10 nmol (1 to 2 μg) of each protein were combined into a 50-μL reaction in an inert reaction buffer without any phosphate and with or without the substrate seduheptulose-7-phosphate (S7-P; buffer containing 20 mM HEPES [pH 7.5], 5.8 mM ATP, 20 mM KCl, and 10 mM $MgCl_2$); reaction mixtures were incubated overnight (approximately 18 h) at 37°C. After heat inactivation (HI), this material was directly used for all cell coincubations. For NMR analysis, the samples were boiled to inactivate any proteins or enzymes and precipitated with methanol (1:3 [vol/vol]; 1 volume reaction mixture to 3 volumes methanol) to remove denatured protein. After protein removal by centrifugation, the supernatant containing the metabolite reaction products was then concentrated to dryness by a flow of gaseous nitrogen. For mass spectrometry, the reaction mixtures were scaled up to 300-μL volumes.

NMR and mass spectrometry analysis of bacterial heptose metabolites and reaction products. (i) Reference ADP-heptose for NMR analysis. Fifty microliters of each commercial reagent, β-D-ADP-heptose (2 mM; Invivogen, France), and β-L-ADP-heptose (J&K, China), was dried under a stream of nitrogen and dissolved in 120 μL of heavy water (D_2O). These were used as references for the chemical identity of *H. pylori*-produced ADP-heptose isomers.

(a) Sample preparation. The following samples were prepared for NMR analysis of heptose metabolites (compare also Table 3): 2, control sample containing 20 mM HEPES buffer at pH 7.5 (20 mM KCl, 10 mM $MgCl_2$, and 6 mM NaATP) and *H. pylori* purified pathway enzymes GmhA, HldE, and GmhB but no pathway substrate; 6, HldE and substrate seduheptulose-7-phosphate (S7-P; 2 mM; Sigma-Aldrich), putative product is S7-P; 11, enzymes GmhA and HldE and substrate S7-P (2 mM, Sigma-Aldrich), putative product is β-D-heptose-1,7-bisphosphate (D-glycero-β-D-manno-heptose-1,7-biphosphate [β-D-HBP]); 9, enzymes GmhA, HldE, and GmhB and substrate S7-P (2 mM), putative product is ADP-heptose. All samples were incubated for metabolite biosynthesis at 37°C overnight and subsequently heat inactivated at 95°C for 20 min. Afterward, all samples were treated with methanol (3:1 methanol:samples) for protein precipitation and pelleted by centrifugation. Each of the supernatants was dried under a stream of N_2 at about 40°C for 30 min, and the remaining residue was dissolved in 120 μL of D_2O. Each of the solutions was carefully filled into a Bruker NMR Match microtube.

(ii) NMR spectroscopy for metabolite identification and characterization. All NMR spectra were recorded with a Bruker AVANCE 500 MHz spectrometer equipped with an solid electrolyte interface (SEI) probe using TopSpin version 3.5 (Bruker Biospin, GmbH, Rheinstetten, Germany). ^{1}H NMR spectra were recorded with the Bruker pulse program "noesygppr1d" for suppression of the water signal during the relaxation period, applying a narrow saturation pulse with a line width of about 25 Hz. The parameters were ns = 64 or 256, ds = 8 s, TE = 25°C, aq = 2.73 s, td = 32,768, sw = 12.0 ppm, and p1 = 8.20 μs. ^{1}H,^{1}H-COSY spectra with water suppression were recorded with the Bruker pulse program "cosygpprqf" with ns = 4 or 64, ds = 8 s, TE = 25°C, aq = 0.10 s, td = 1,024 (f2), 256 (f1), sw = 10.00 ppm, and p1 = 8.20 μs. Data were processed with MestreNova version 14.2.0 (Mestrelab Research, Santiago de Compostela, Spain). The flame ionization detections (FIDs) were zero filled and multiplied by a mild Gaussian function before Fourier transformation. Under these conditions, the detection limit for ADP-heptose was approximately 27 μg of dissolved ADP-heptose, that is, 361.8 μM (applying 128 scans in the one-dimensional experiment). The ^{1}H-NMR spectrum of 830 μM ADP-heptose is shown in Fig. 6E, 6F. Results for other reference samples are documented in Fig. 6A through 6D. With the help of ^{1}H,^{1}H-COSY and by comparing to previously published data (42, 54), the detected signals could be clearly assigned to protons of the adenine unit, the ribose unit, and the heptose unit. Further on, characteristic CH_3 signals at 1.21 ppm and CH_2 signals at 3.14 ppm belonging to trimethylamine were detected for the reference compound. Notably, triethylamine is often used during the chemical synthesis of ADP-heptose as a protection reagent and is present as the counter ion in the reference sample (8). Chemical shifts, multiplicity, coupling constants, and correlations observed in the ^{1}H,^{1}H-COSY spectrum for substrates, reference compounds (S7-P, β-D-ADP-heptose, and β-L-ADP-heptose), and products are presented in Table S3.

(iii) Mass spectrometry (LC-ESI MS/MS) of heptose metabolites. Pure reagents, *in vitro* reaction mixtures, or cell extracts were analyzed on an Agilent 6495 triple quadrupole mass quadrupole mass spectrometer equipped with an ESI ion source and coupled to an Agilent 1290 Infinity II ultrahigh performance liquid chromatographer (UHPLC; both Agilent Technologies). Chromatographic separation of ADP-heptose was achieved with an Acquity ultraperformance liquid chromatography (UPLC) BEH amide (1.7 μm, 2.1 $\times$ 100 mm) column (Waters). Solvent A consisted of water with ammonium formate (10 mM) and formic acid (0.1% vol/vol). Solvent B consisted of acetonitrile with formic acid (0.1% vol/vol). The LC gradient was as follows: 0 min 90% B, 7 min 40% B, 8 min 40% B, 8.5 min 90% B, and 12 min 90% B (flow rate of 0.1 mL/min). The heptose-phosphates were separated by a SeQuant ZIC-pHILIC (5 μm, 150 $\times$ 2.1 mm) column (Merck). Solvent A consisted of water with ammonium carbonate (10 mM) and ammonium hydroxide (0.2%). Solvent B consisted of acetonitrile. The LC gradient was as follows: 0 min 90% B, 12 min 40% B, 14 min 40% B, 15 min 90% B, and 18 min 90% B (flow rate of 0.2 mL/min). Three microliters was injected per sample. The settings of the ESI source were 200°C source gas temperature, 14 L/min drying gas, and 24 lb/in^2 nebulizer pressure. The sheath gas temperature was 250°C, and the flow was 11 L/min. The electrospray nozzle was set to 500 V, and capillary voltage was 2,500 V. ADP-heptose was analyzed in positive ion mode with a transition from 620 *m/z* to 428 *m/z* (collision energy of 10 keV and a dwell time of 100 ms). Heptose-bisphosphate was analyzed in positive ion mode with a transition from 371 *m/z* to 273 *m/z* (collision energy of 10 keV and dwell time of 100 ms). Heptose-monophosphates were analyzed in negative ion mode with a transition from 289 *m/z* to 79 *m/z* (collision energy of 40 keV and dwell time of 100 ms). Raw data were converted into text files using MSConvert (55). Data analysis was performed with a customized MATLAB script. Bioblanks were also measured by spiking the reference compounds into bacterial lysates to determine possible shifts in retention times.

Data availability. Full RNA-seq results are accessible on NCBI's GEO under accession number GSE227450.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.6 MB.

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Supplements to Publication II:

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Supplemental materials accessible via:

https://journals.asm.org/doi/10.1128/spectrum.03132-22?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%20%200pubmed

Files:

spectrum.03132-22-s0001.pdf (see below)

spectrum.03132-22-s0002.xlsx (large supplemental file Table S1, contained on USB device)

spectrum.03132-22-s0003.xlsx (large supplemental file Table S2, contained on USB device)

Supplemental Materials

Helicobacter pylori modulates core LPS heptose biosynthesis and heptose-dependent innate immune host cell activation by multiple mechanisms

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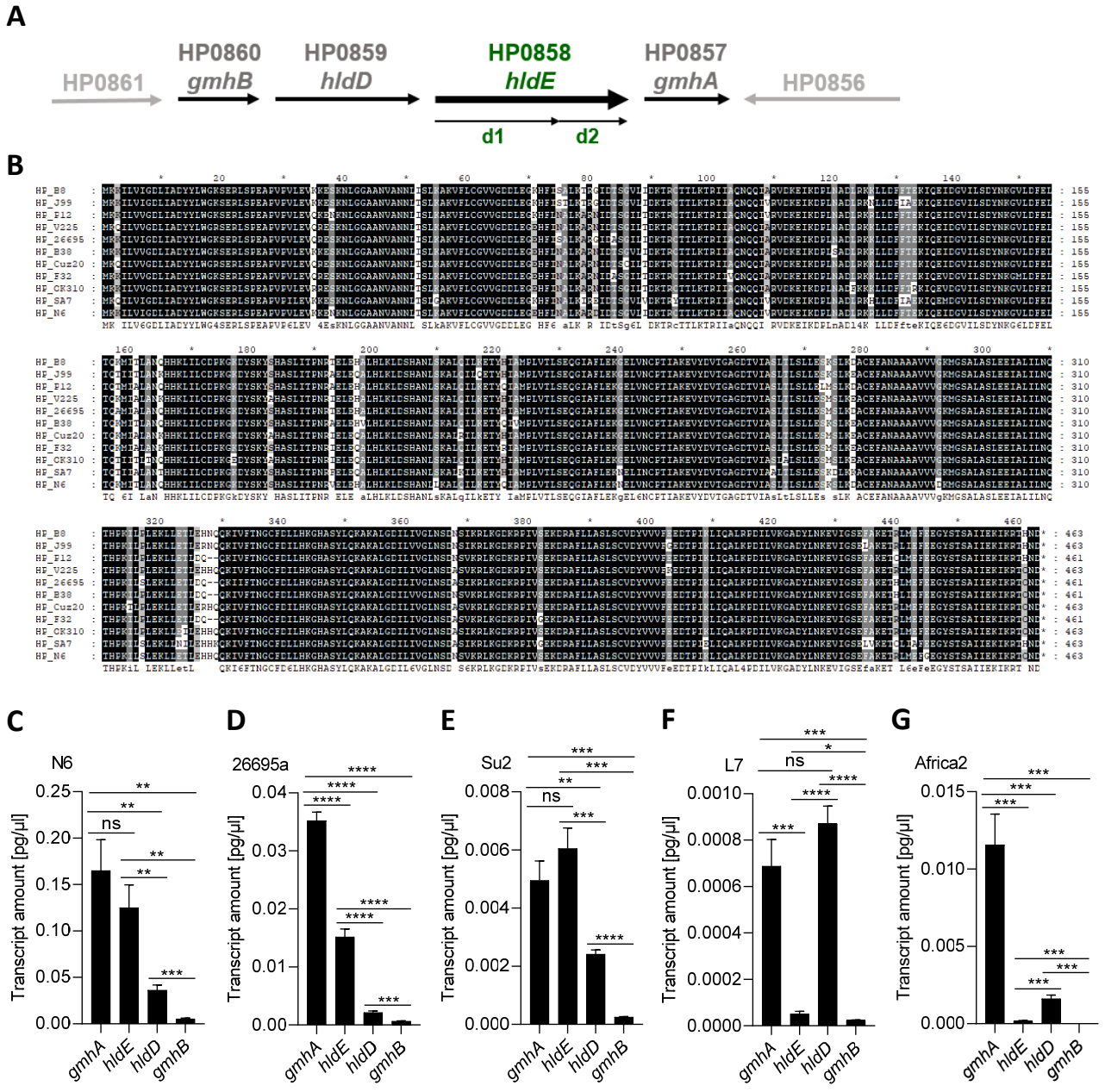

Fig S1. Genomic arrangement, protein diversity and strain-specific regulation in the *H. pylori* heptose biosynthesis gene cluster. A) Organisation of heptose biosynthesis operon genes HP0861 to HP0857/*gmhA* in *H. pylori* 26695a. *hldE* is highlighted in green color. Gene arrangement in the cluster is conserved in other strains isolated worldwide. **B)** variation of HP0858/HldE protein sequence in diverse *H. pylori* strains (B8, J99, V225, 26695, B38, Cuz20, F32, OK310, SA7, HPN6), selected from different geographic origins and strain populations. Note the very conspicuous hinge region in HldE between the two domains d1 and d2, characterized by gaps in some strains. **C)** to **G)** depict quantification of transcript amounts of heptose biosynthesis cluster genes *gmhA, hldE, hldD,* and *gmhB* of *H. pylori* wild type strains N6 (C), 26695a (D), Su2 (E), L7 (F) and Africa2 (G) by qPCR, performed in technical triplicates. All qPCR results, given in absolute quantities of pg/ml, were normalized to 16S rRNA transcript amounts of each sample. Pairwise significance of differences (p values) in panels C) through G) was calculated by unpaired student's *t*-test. Significance values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns = non-significant.

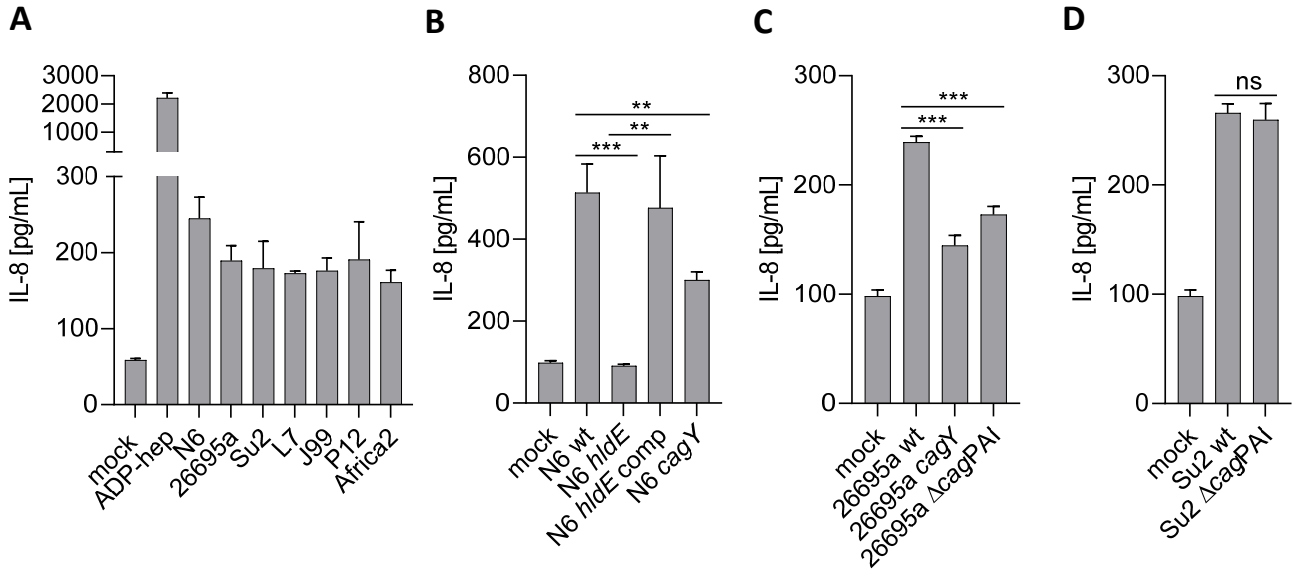

Fig S2. Gastric epithelial cell line MKN28 response to *H. pylori* ETLs. A) to D) Activation of MKN28 gastric epithelial cells after co-incubation with ETLs produced from various *H. pylori* wild type (wt) strains (A) and mutants (B-D) as indicated on the x-axis, for 4 h. A quantitative read-out for pro-inflammatory response was obtained by performing IL-8 ELISA. Shown are the results from technical triplicates of biological duplicates. All experiments were repeated at least once on two different days, with similar results. For B), C) and D), statistical significance was calculated for differences between wt strain and each mutant or complemented strain. Significance of differences (p values) was calculated by unpaired student‘s *t*-test. Significance values: ** $p < 0.01$; *** $p < 0.001$; ns = non-significant. In all cell activation experiments, co-incubation with pure ADP-heptose (2.5 µM, shown in A)) served as a reference for activation.

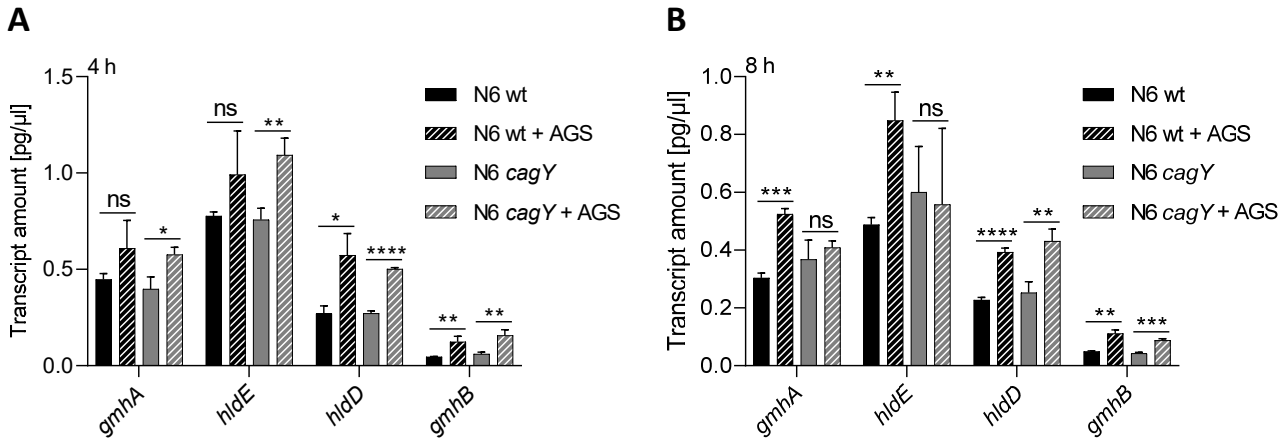

Fig S3. Time-dependent regulation of heptose biosynthesis gene cluster in *H. pylori* N6 and its isogenic *cagY* mutant, co-incubated with gastric epithelial AGS cells. A) and **B)** show transcript amounts (RT-qPCR) of heptose cluster genes *gmhA*, *hldE* (HP0858), *hldD*, *gmhB* (HP0860) of *H. pylori* N6 wild type and *cagY* (HP0527) mutant, both co-incubated in the presence or absence of AGS cells (MOI=50) for 4 h (A) or 8 h (B), respectively. Control bacteria were incubated in cell culture medium alone for the respective time periods. Three technical replicates are summarized in the panels. All qPCR results, shown in absolute transcript amounts of pg/µl, were normalized to 16S rRNA transcript amounts of each sample. Statistically significant differences (p) between conditions were calculated by unpaired student's *t*-test. Significance values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns = non-significant.

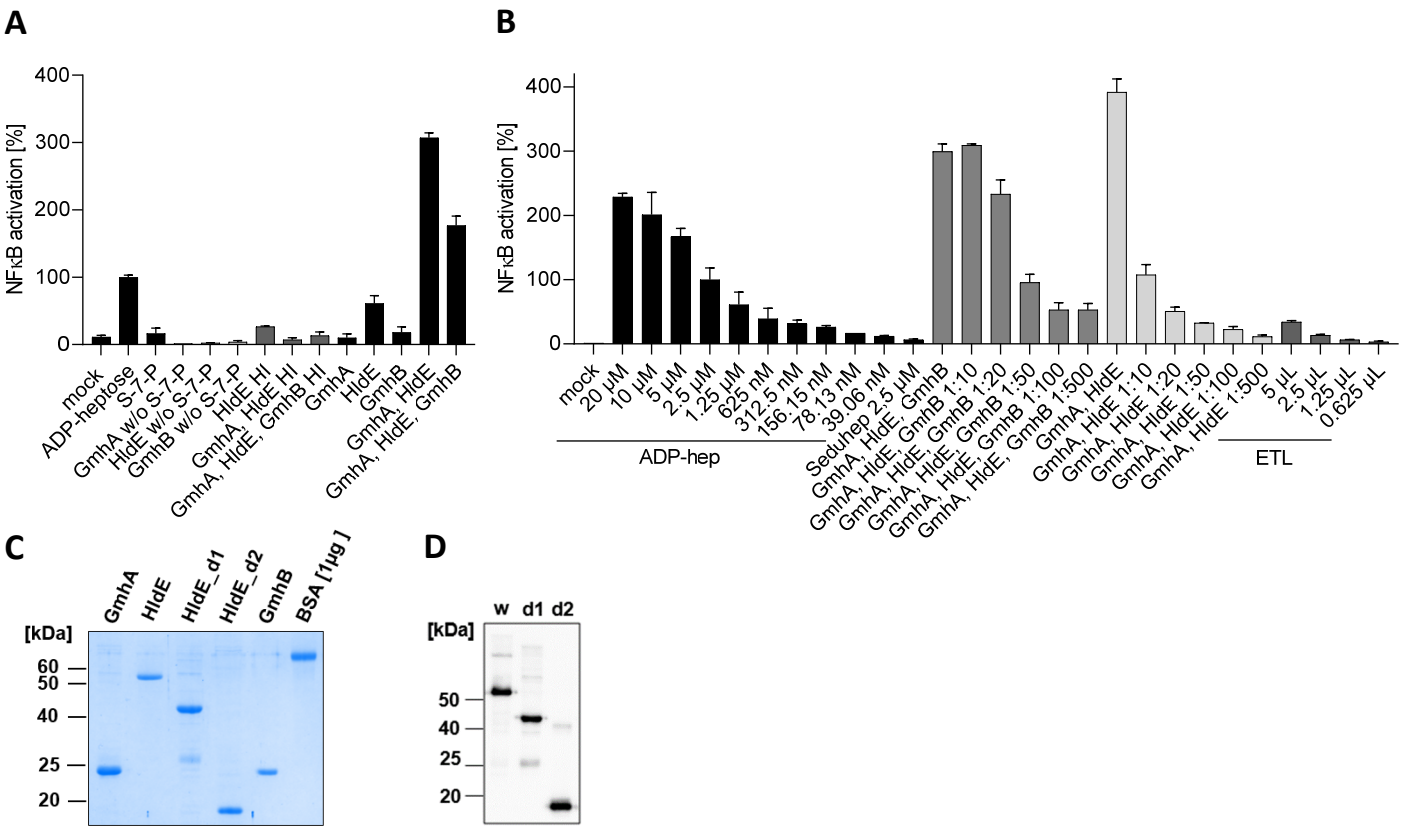

Fig S4. Quality controls of purified recombinant *H. pylori* heptose biosynthesis enzymes and activity titrations for in vitro reconstitution of heptose biosynthesis pathway.

A) Activation of NF-κB reporter cells (HEK_luc) by heptose metabolites synthesized using one-pot reactions of recombinant *H. pylori* 26695a proteins with enzymatic substrate seduheptulose 7-phosphate (S-7-P), and respective controls. Controls include active single enzymes plus substrate, or heat-inactivated (HI) enzymes plus substrate. **B)** Activation of – HEK NF-κB luciferase reporter cells by pure β-D-ADP-heptose (titration), the products of three-enzyme combination GmhA, HldE and GmhB, the reaction product of two-enzyme combination GmhA, HldE (*in vitro* reconstitution: in each case non-diluted reaction sample and the same used at different dilutions from 10^{-1} to $5x10^{-2}$). In addition, ETL (enzymatically treated lysate) prepared from strain N6 with an OD_{600} of 2/ml of the original culture, was used. All reporter assays were performed in technical triplicates and repeated at least once independently (biological replicates) on different days. **C)** Protein quality control SDS gel showing Ni^{2+}-NTA-purified recombinantly expressed heptose biosynthesis enzymes, GmhA, HldE (and its separate d1 and d2 domains), GmhB, all from *H. pylori* 26695a. All proteins (bands detectable at predicted masses) show a purity of >95%. HldE cloned from strain N6 was purified with similar quality features (not shown). **D)** Western immunoblot detecting purified 6xHis-HldE and separately expressed 6xHis HldE d1 and d2 domains (cloned from strain 26695a), using a custom-produced HldE antibody. The immunoblot detection demonstrates that the custom-produced antibody (1:20.000, rabbit) recognizes the full-length HldE protein and both separate HldE domains. The three main bands detected are full-length HldE (w, 52 kDa), its N-terminal domain (d1, 36 kDa), and its C-terminal domain (d2, 15 kDa), expressed separately.

Table S3. Chemical shifts, multiplicity, coupling constants as well as scalar correlations observed in the $^{1}H,^{1}H$-COSY spectrum for substrates, reference compounds and combined samples (*in vitro* reconstitution mixes of *H. pylori* heptose biosynthesis enzymes). Table see next page.

		^{1}H NMR results (this study)				Published data (Literature)		
Reference or Sample Name	Position	δ ^{1}H [ppm]	Multiplet	J [Hz]	^{1}H,^{1}H-COSY	δ ^{1}H [ppm]	Multiplet	J [Hz]
Reference AMP	H1'-AMP	6.05	d	6.2	H2'-AMP			
	H2'-AMP	4.70	-	-	H1'-AMP, H3'-AMP			
	H3'-AMP	4.42	m	-	H2'-AMP, H4'-AMP			
	H4'-AMP	4.28	m	-	H3'-AMP, H5'-AMP			
	H5'-AMP	3.94	m	-	H4'-AMP			
	H2''-AMP	8.17	s	-	-			
	H8''-AMP	8.52	s	-	-			
Reference ADP	H1'-ADP	6.07	d	5.50	H2'-ADP			
	H2'-ADP	4.67	-	-	H1'-ADP, H3'-ADP			
	H3'-ADP	4.49	m	-	H2'-ADP, H4'-ADP			
	H4'-ADP	4.32	m	-	H3'-ADP, H5'-ADP			
	H5'-ADP	4.17	m	-	H4'-ADP			
	H2''-ADP	8.17	s	-	-			
	H8''-ADP	8.44	s	-	-			
Reference ATP	H1'-ATP	6.06	d	5.70	H2'-ATP			
	H2'-ATP	4.70	-	-	H1'-ATP, H3'-ATP			
	H3'-ATP	4.48	m	-	H2'-ATP, H4'-ATP			
	H4'-ATP	4.33	m	-	H3'-ATP, H5'-ATP			
	H5'-ATP	4.19	m	-	H4'-ATP			
	H2''-ATP	8.18	s	-	-			
	H8''-ATP	8.43	s	-	-			
Reference ADP-Heptose (D-form) (Invivogen)	H1'-Ribose	6.08	d	6.0	H2'-Ribose	6.16	d	5.8
	H2'-Ribose	4.67	m	-	H1'-Ribose, H3'-Ribose	4.73	m	4.9
	H3'-Ribose	4.46	dd	3.5 / 5.2	H2'-Ribose, H4'-Ribose	4.54	dd	3.8
	H4'-Ribose	4.33	m	-	H3'-Ribose, H5'-Ribose	4.41	m	-
	H5'-Ribose	4.16	dd	3.0 / 5.3	H4'-Ribose	4.23	m	-
	H2''-Adenine	8.20	s	-	-	8.29	-	-
	H8''-Adenine	8.44	s	-	-	8.53	-	-
	H1-Heptose	5.13	dd	1.0 / 8.7	n.d.	5.21	dd	1.0/8.7
	H2-Heptose	3.99	d	3.2	H3-Heptose	4.07	dd	3.3
	H3-Heptose	3.56	dd	3.2 / 9.5	H2-Heptose	3.63	dd	9.4
	H4-Heptose	3.60	m	-	H5-Heptose	3.69	t	9.4

	H5-Heptose	3.37	dd	3.3 / 9.7	H4-Heptose, H6-Heptose	3.45	dd	3.3
	H6-Heptose	3.92	m	-	H5-Heptose, H7-Heptose	3.99	m	-
	H7-Heptose	3.67	m	-	H6-Heptose	3.75	m	-
	H1-HMP-1[#]	4.98	dd	1.1/8.8	n.d.	5.08	-	-
	H2-HMP-1	3.91	m	-	n.d.	4.01	-	-
	H3-HMP-1	n.d.	n.d.	n.d.	n.d.	3.69	-	-
	H4-HMP-1	3.60	m	-	H5-HMP-1	3.79	-	-
	H5-HMP-1	3.42	dd	3.0/9.9	H4-HMP-1	3.49	-	-
	H6-HMP-1	3.93	m	-	H7-HMP-1	4.03	-	-
	H7-HMP-1	3.70	m	-	H6-HMP-1	3.79/3.69	-	-
Reference ADP-Heptose (L-form) (J&K)	H1'-Ribose	6.08	d	6.0	n.d.	6.18	d	5.8
	H2'-Ribose	n.d.	n.d.	n.d.	n.d.	4.70	m	5.2
	H3'-Ribose	4.46	dd	3.5/5.2	H4'-Ribose	4.55	dd	3.5
	H4'-Ribose	4.33	m	-	H3'-Ribose, H5'-Ribose	4.42	m	-
	H5'-Ribose	4.15	m	-	H4'-Ribose	4.25	m	-
	H2''-Adenine	8.20	s	-	-	8.37	-	-
	H8''-Adenine	8.44	s	-	-	8.60	-	-
	H1-Heptose	5.15	dd	1.0/8.3	n.d.	5.23	dd	1.0/8.5
	H2-Heptose	4.00	d	3.3	H3-Heptose	4.07	dd	3.2
	H3-Heptose	3.60	dd	3.3/9.9	H2-Heptose, H4-Heptose	3.67	dd	9.7
	H4-Heptose	3.67	m	-	H3-Heptose, H5-Heptose	3.82	t	9.7
	H5-Heptose	3.28	dd	1.7/9.9	H4-Heptose,	3.37	dd	1.7
	H6-Heptose	3.87	m	-	H7-Heptose	3.95	ddd	6.0/5.0
	H7-Heptose	3.73	t	9.9	H6-Heptose	3.75/3.71	dd	12.3
Sample 9[§]	H1'-Ribose	6.08	d	6.0	H2'-Ribose	6.16	d	5.8
	H2'-Ribose	4.70	m	-	H1'-Ribose, H3'-Ribose	4.73	m	4.9
	H3'-Ribose	4.45	m	-	H2'-Ribose, H4'-Ribose	4.54	dd	3.8
	H4'-Ribose	4.32	m	-	H3'-Ribose, H5'-Ribose	4.41	m	-
	H5'-Ribose	4.16	m	-	H4'-Ribose	4.23	m	-
	H2''-Adenine	8.19	s	-	-	8.29	-	-
	H8''-Adenine	8.43	s	-	-	8.53	-	-
	H1-Heptose	5.13	dd	1.0 / 8.7	-	5.21	dd	1.0/8.7
	H2-Heptose	3.99	d	3.3	H3-Heptose	4.07	dd	3.3
	H3-Heptose	3.55	dd	3.3 / 9.5	H2-Heptose	3.63	dd	9.4
	H4-Heptose	3.61	m	-	H5-Heptose	3.69	t	9.4

	H5-Heptose	3.37	dd	3.3 / 9.5	H4-Heptose	3.45	dd	3.3
	H6-Heptose	3.93	m	-	H7-Heptose	3.99	m	-
	H7-Heptose	3.70	m	-	H6-Heptose	3.75	m	-
Sample 11[§]	H1-HMP-1	5.02	dd	1.1/8.6	-	5.08	-	-
	H2-HMP-1	3.90	-	-	-	4.01	-	-
	H3-HMP-1	3.55	-	-	-	3.69	-	-
	H4-HMP-1	3.60	-	-	-	3.79	-	-
	H5-HMP-1	-	-	-	-	3.49	-	-
	H6-HMP-1	3.95	-	-	-	4.03	-	-
	H7-HMP-1	3.69	-	-	-	3.79/3.69	-	-
Sample 9d1[§]	H1-HMP-1	4.97	dd	1.0/8.8	H2-HMP-1	5.08	-	-
	H2-HMP-1	3.90	m	-	H1-HMP-1, H3-HMP-1	4.01	-	-
	H3-HMP-1	3.59	m	-	H2-HMP-1	3.69	-	-
	H4-HMP-1	n.d.	-	-	-	3.79	-	-
	H5-HMP-1	3.39	m	-	H6-HMP-1	3.49	-	-
	H6-HMP-1	3.93	m	-	H5-HMP-1, H7-HMP-1	4.03	-	-
	H7-HMP-1	3.69	m	-	H6-HMP-1	3.79/3.69	-	-
Sample 9d1d2[§]	H1'-Ribose	6.08	d	6.0	H2'-Ribose	6.16	d	5.8
	H2'-Ribose	4.70	m	-	H1'-Ribose, H3'-Ribose	4.73	m	4.9
	H3'-Ribose	4.45	m	-	H2'-Ribose, H4'-Ribose	4.54	dd	3.8
	H4'-Ribose	4.32	m	-	H3'-Ribose, H5'-Ribose	4.41	m	-
	H5'-Ribose	4.16	m	-	H4'-Ribose	4.23	m	-
	H2''-Adenine	8.19	s	-	-	8.29	-	-
	H8''-Adenine	8.43	s	-	-	8.53	-	-
	H1-Heptose	5.11	dd	1.0 / 8.7	-	5.21	dd	1.0 / 8.7
	H2-Heptose	3.98	d	3.3	H3-Heptose	4.07	dd	3.3
	H3-Heptose	3.54	dd	3.3 / 9.5	H2-Heptose	3.63	dd	9.4
	H4-Heptose	3.59	m	-	H5-Heptose	3.69	t	9.4
	H5-Heptose	3.35	dd	3.3 / 9.5	H4-Heptose	3.45	dd	3.3
	H6-Heptose	3.94	m	-	H7-Heptose	3.99	m	-
	H7-Heptose	3.70	m	-	H6-Heptose	3.75	m	-

[§] ATP, ADP, and AMP signals in non-reference samples are not separately listed. # Reference compound β-D-ADP-heptose (commercial source: Invivogen) contained a small amount of β-HMP-1 (boxes shaded in light grey)

Supplemental Literature:

ADP-heptose:

Zamyatina A, Gronow S, Puchberger M, Graziani A, Hofinger A, Kosma P. Efficient chemical synthesis of both anomers of ADP L-glycero- and D-glycero-D-manno-heptopyranose. *Carbohydr Res*. **2003**, 338(23), 2571-2589.

HBP/HMP:

Adekoya IA, Guo CX, Gray-Owen SD, Cox AD, Sauvageau J. D-Glycero-β-d-Manno-Heptose 1-Phosphate and d-Glycero-β-d-Manno-Heptose 1,7-Biphosphate Are Both Innate Immune Agonists. *J Immunol*. **2018,** 201(8), 2385-2391.

4. Discussion

Living persistently in the stomach of its host for a life span, if not treated, makes *H. pylori* an exceptional successful colonizer (Suerbaum and Josenhans 2007). To achieve this, *H. pylori* is highly adapted to its unstable environment due to fast mutation rates (Kuipers, Israel et al. 2000, Ailloud, Didelot et al. 2019). During to the close association to its host (Schreiber, Konradt et al. 2004) *H. pylori* needs to interact with its host and has evolved ways to modulate host responses (Suerbaum and Josenhans 2007, Faass, Hauke et al. 2023 A). The activation of host immune responses by heptose metabolites is only one way in which *H. pylori* interacts with its host. At the beginning of this work, it was established that heptose metabolites, and ADP-heptose in particular, act as MAMPs in various bacteria, including *H. pylori*. In addition, the heptose signaling pathway had been elucidated by extensive studies of host factors, however, was almost exclusively shown for epithelial host cells before. Despite the high amount of work that many groups, including ours, have performed on heptose-dependent signaling over the last years, and the consolidated fundamental knowledge about the bacterial heptose biosynthetic pathway (Kneidinger, Marolda et al. 2002), many questions remain unresolved. In this thesis, we first investigated the potential of heptose metabolites to activate other host cells besides epithelial cells as well, by working with cells of the phagocytic lineage (Publication I). Further, we aimed to elucidate how the heptose biosynthesis and thus the heptose-dependent pro-inflammatory signaling is regulated in our model organism *H. pylori*. The great diversity of *H. pylori* suggested the possibility of strain-specific regulation and production of heptose metabolites. Therefore, in Publication II, regulation and synthesis of heptose metabolites were investigated in a strain-specific manner. Both Publications I and II, investigate strain-specific heptose signaling, with Publication I focusing on the activation of phagocytic cells. With our recent findings, we shed light onto the activation potential of various heptose metabolites on different host cells, the complex regulation of the biosynthesis and the biochemical characterization of the bacterial heptose products. Fig. 2 provides a model about how we can now summarize that *H. pylori* regulates the synthesis of various heptose metabolites and uses them to interact with its host. In this section, our new findings will be further discussed and integrated into the current knowledge about heptose metabolites of *H. pylori* and other bacteria and the use of these pro-inflammatory stimulants to modulate host infection and interaction.

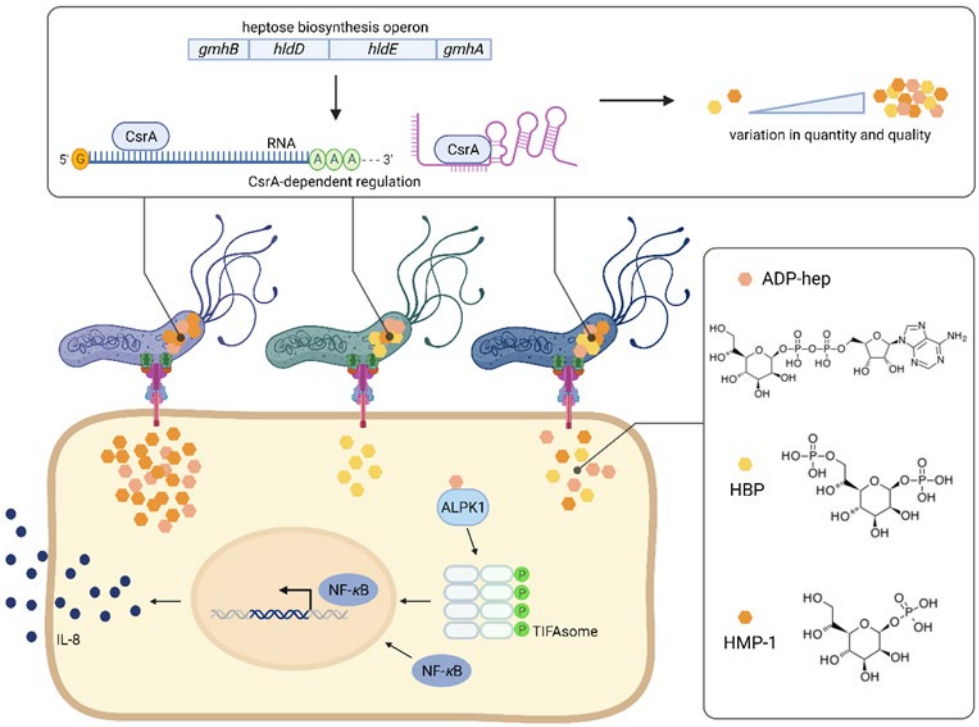

Fig. 2. Our current model of how heptose production and transport capacities of *H. pylori* are involved in the variable pro-inflammatory activation of host cells. In this model, the various heptose metabolites ADP-heptose, HBP and HMP-1 are produced by *H. pylori* which induce a CagT4SS-dependent host response resulting in ALPK1-TIFA-NF-κB signaling. This is a universal pro-inflammatory mechanism, which involves the PRR ALPK1, TIFA-formation and NF-κB activation, for many different cell types (Publication I). The signaling cascade leads to the release of cytokines and the recruitment of immune cells, reviewed by García-Weber and Arrieumerlou (2021). Our study investigating the variation of putative metabolites provided solid evidence for the production and structure of different immune-active metabolites (Publication II). Structures of the pro-inflammatory immune active heptose metabolites and colors of the respective symbols (right panel) are as in Figure 1). Further, the results obtained in this work suggests adaptation of heptose production in response to environmental influences by up- or downregulation of the heptose operon which includes also the possibility of upon-demand production of metabolites in close proximity to host cells. Comprehensive transcriptome analysis and follow up experiments, suggest involvement of the global regulator CsrA in heptose operon regulation (Publication II). CsrA can act as regulatory protein via different mechanisms, impacting translation, RNA abundance and RNA stability of a variety of target genes and was shown to have important regulatory functions in various bacterial species (Vakulskas, Potts et al. 2015, Potts, Vakulskas et al. 2017). Control of CsrA in *H. pylori* may lead to a variation of heptose metabolites in quantity and composition, which according to the new evidence in the present thesis, also vary in a strain-specific manner. Factors involved in the regulation of the production of various heptose metabolites are shown in the top panel. The figure was created using BioRender.com.

4.1 *H. pylori* heptose metabolites activate myeloid cells in addition to epithelial cells, aided by the CagT4SS

4.1.1 Heptose-dependent activation is of universal importance for the interaction of *H. pylori* with different types of host cells

While heptose metabolites have been classified as MAMPs as they elicit an immune response in epithelial cells, activation of other types of host cells by heptoses has received little attention. During infection, the first line of defense is the physical barrier built by epithelial cells (Günther and Seyfert 2018). Gastric epithelial cells sense infection through various receptors and recruit cells of the myeloid lineage to the site of infection (Medzhitov 2007). Following disruption of the epithelial barrier, which can be caused by *H. pylori* infection (Noach, Rolf et al. 1994, Wroblewski and Peek 2011), these myeloid cells can be found in the gastric mucosa. Fehlings and colleagues demonstrated the presence of activated macrophages in the stomach mucosa of *H. pylori* infected individuals and in addition, neutrophils and dendritic cells have been found (Fehlings, Drobbe et al. 2012, Fu, Ma et al. 2016). The recognition and interaction of *H. pylori* with myeloid cells was also reviewed by our group (Faass, Hauke et al. 2023 A), and summarized findings support the existence of a direct interaction surface between *H. pylori* and myeloid cells. Koch and colleagues found that macrophages were activated by *cag*PAI-positive *H. pylori* strains independent of effector CagA and TLR signaling (Koch, Mollenkopf et al. 2016). In retrospect, these results suggest heptose-dependent signaling, however, could not yet be attributed to heptose signaling at this time. Although highly relevant in the context of *H. pylori* infection, knowledge about heptose-dependent signaling and myeloid cells was still lacking. Therefore, we sought to fill this gap of knowledge by focusing on the response of macrophages to heptose metabolites (Publication I). Heptose-dependent activation of macrophages (cell culture and primary cells) was demonstrated by NF-*κ*B-activation, secretion of cytokines and the transcription level of inflammation-associated genes (Publication I). Our additional study shows that this is also the case in neutrophils, as they react to pure ADP-heptose despite not being activated by common ligands (Faass, Hauke et al. 2023 B). Further the study includes and compares transcriptome analysis of the three cell types, gastric epithelial cells, macrophages (also in Publication I) and neutrophils, in terms of their response upon co-incubation with pure heptose metabolites (Faass, Hauke et al. 2023 B). Comparison of differentially expressed genes of the three cell types showed a strong overlap. Together with the results of Publication I, we can now provide a good overview of the response of different cell types to bacterial heptose metabolites, all showing strong immune responses with some similarities (Faass, Hauke et al. 2023 A).

They were also strongly influenced by a functional CagT4SS, suggesting that active transport of heptose metabolites is required in all target cell types. The cell type-specific responses and transcriptomes will need to be analyzed further. Our work not only contributes to fill the previously mentioned gap of knowledge of heptose signaling in myeloid cells, but also provides strong evidence for the universal importance of the activation by bacterial heptose metabolites. In conclusion, the findings highlight the importance of heptose signaling in the inflammation scenario, which also involves myeloid cells like macrophages and neutrophils, and help to better understand the modulation of host-pathogen interactions by *H. pylori*.

4.1.2 *H. pylori* uses different heptose metabolites to provoke pro-inflammatory signaling

In an attempt to elucidate heptose-dependent signaling and associated host factors in *H. pylori*, several bacterial metabolites have been found before. Work by several groups pointed to HBP as possible immune-active metabolite in *H. pylori*, but the identity of any potential heptose metabolite could not be confirmed so far (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). Prior to this thesis, the important study of Adekoya et al. (2018) compared the immune activation potential of several chemically synthesized metabolites. This included HMP-1, the metabolite one step further downstream in the heptose biosynthesis cascade from HBP. HMP-1 showed potent immune activation comparable to the activation potential of HBP when co-incubated with epithelial cells adding another putative active metabolite to the list (Adekoya, Guo et al. 2018). In parallel, other studies provided evidence that ADP-heptose (Zhou, She et al. 2018) is the predominant metabolite in *H. pylori* (Pfannkuch, Hurwitz et al. 2019). Even taking together all attempts to identify the immune active heptose metabolites in *H. pylori*, a clear or generalized statement cannot be made yet. Therefore, a major goal of this work was to clarify which immune-active metabolites are produced by *H. pylori*. By reconstituting the heptose biosynthesis pathway *in vitro*, we demonstrated that *H. pylori* enzymes can indeed produce pro-inflammatory active products other than ADP-heptose (Publication II). Those were biochemically identified in *H. pylori* and by *in vitro*-reconstitution as HBP and HMP-1 (Publication II). The question now arises how our results are compatible with previous findings on the identity of heptose metabolites in *H. pylori* and other Gram-negative bacteria. Our results demonstrate (Publication II) pro-inflammatory activation via ALPK1-TIFA signaling of an *in vitro*-synthesized metabolite even when only using the first two enzymes of the cascade together with the substrate S-7-P. In parallel to our work, *in vitro*-reconstitution was performed for the heptose pathway of *C. jejuni* as well, similarly demonstrating

immune activation potential of the metabolite produced using the first two enzymes of the pathway (Cui, Duizer et al. 2021). This suggests that *H. pylori*, and possibly also other bacteria, produce other active metabolites in addition to ADP-heptose. This finding is in contrast to the conclusion of Pfannkuch et al. that ADP-heptose is the predominant metabolite, as they found only small amounts of HBP in *H. pylori* lysates (Pfannkuch, Hurwitz et al. 2019). Since previous work, including studies on other bacteria, hardly contributed to the knowledge about the composition of metabolites in bacterial lysates, we sought to analyze these as well using different *H. pylori* strains. Even after considerable efforts, we had difficulty in biochemically dissecting lysates content of *H. pylori*, which tought us that this is a challenging task, where metabolites, e.g. HMP-1 due to its mass similarity to the non-active precursor HMP-7, may be easily overlooked. For the identification we used mass spectrometry and NMR, as the distinction of HMP-1 and HMP-7 was initially better in NMR. In addition to this complexity, the study by Pfannkuch et al. involved only one *H. pylori* strain, P12. As we can demonstrate for the first time (Publication II), heptose metabolite quantity and quality differs between *H. pylori* strains. *H. pylori* P12 produced a high amount of ADP-heptose compared to six other wild type strains, which raises the question of whether this particular strain may not produce much HBP because of the high ADP-heptose content. In contrast, other strains may produce greater amounts of HBP, serving as possible explanation to the controversial findings. Furthermore, the *gmhB*-defective mutant of *H. pylori* P12 exhibited in a complete loss of immune activity (Pfannkuch, Hurwitz et al. 2019), whereas this was not the case in previous work of our lab using strain N6 (Stein, Faber et al. 2017). Lysate of the *H. pylori* N6 *gmhB*-defective mutant in our study still activates epithelial cells, with a huge increase upon transfection, indicating the presence of a mixture of permeable and non-permeable metabolites other than ADP-heptose, as mass spec confirms that ADP-heptose is not present in this lysate (Publication II). This strain diversity in heptose metabolites underlines the difficulties of generalized findings when studying only one strain. In addition, Zhou et al. stated that, in their own tests, other metabolites than ADP-heptose, HBP and HMP-1 only inefficiently activated receptor ALPK1. By biochemically analyzing ALPK1, expressed and purified from *E. coli*, they demonstrate ADP-heptose to be the structurally best-matching ligand, rather than HBP or HMP-1 (Zhou, She et al. 2018). Injection of ADP-heptose and HBP into mice showed extensive neutrophil recruitment for ADP-heptose but not for HBP (Zhou, She et al. 2018). These results are in strong contrast to investigations on the activation potential of HBP and HMP-1 by Adekoya and colleagues (Adekoya, Guo et al. 2018), which were prepared in parallel to the study by Zhou and colleagues. Here, they clearly demonstrate that the alternative metabolites induce TIFA-dependent signaling in a

similar manner, expanding the active spectrum from ADP-heptose to other metabolites (Adekoya, Guo et al. 2018). Interestingly, in an attempt to solve the question how alternative metabolites are able to activate host cells, Zhou et al. also tested whether it may be a modification of HBP or HMP-1 that allows binding to the receptor kinase. For HBP, they propose a host adenyltransferase that might be able to convert HBP to ADP-heptose-7-phosphate (ADP-hep-7P). Despite having less binding efficiency than ADP-heptose, ADP-hep-7P was indeed able to bind to ALPK1 to activate the kinase (Zhou, She et al. 2018). Although Zhou and colleagues were able to use the host enzyme to convey the modification *in vitro*, plausibility of this event *in vivo* needs further investigation. In support of the hypothesis of host-enzymatic modification, TIFA formation has been shown to be delayed when host cells are activated by HBP compared to the response to ADP-heptose (García-Weber, Dangeard et al. 2018). The studies investigating possible modifications by host enzymes have predominantly focused on HBP, whereas no such explanation is yet available for HMP-1. In addition, the serendipitous finding of receptor ALPK1 in complex with ligand ADP-heptose (Zhou, She et al. 2018) may have limitations to its informative value regarding other metabolites than ADP-heptose to qualify as ALPK1 ligands. When considering our *in vitro*-reconstitution in Publication II, we detected NF-*κ*B activation also after co-incubation of cells with in vitro reaction products which identified as HMP-1 and HBP (Publication II). Since the non-permeable metabolite HBP cannot be responsible for cellular stimulation without transfection, this effect must be due to the presence of HMP-1. In support of our findings, it seems also possible that HMP-1 plays a role in the heptose-dependent signaling of *N. gonorrhoeae*, looking at older studies of Malott and colleagues (Malott, Keller et al. 2013, Gaudet, Sintsova et al. 2015). In the group's more recent study, HBP was identified using in vitro reconstitution of the synthesis pathway, but bacterial supernatants showed a moderate response before transfection, which was strongly increased after transfection. Again, transfection experiments confirmed that HBP cannot be the only metabolite produced in *N. gonorrhoeae*, since another, permeable, metabolite in lysates has to be responsible for the activation without transfection. HMP-1 could have be present in these samples too, especially considering that only *in vitro*-reconstituted samples were analyzed using mass spectrometry (Gaudet, Sintsova et al. 2015). Two years prior to this, the same group found a heptose metabolite with a mass similar to heptose monophosphate in *N. gonorrhoeae* supernatants (Malott, Keller et al. 2013), which, looking at it in retrospect, supports the conclusion drawn from all data taken together and provides further evidence for the relevance of HMP-1 in other bacteria. Further, our *in vitro*-reconstitution sample containing only the first domain of enzyme HldE showed a strong activation potential on human cells, a sample in which only small

amounts of HBP and large amounts of HMP-1 were detected (Publication II). We can find strong evidence that the immune-activating HMP-1 is produced at high levels in *H. pylori*. This is supported by Adekoya and colleagues who demonstrated a strong pro-inflammatory response of host cells to chemically synthesized HMP-1 (Adekoya, Guo et al. 2018) and the evidence for HMP-1 in reinterpreting active heptose content in *N. gonorrhoeae*. Despite difficulties with the *in vivo* identification and room for further investigations on the alternative active metabolite HMP-1, our data clearly indicate that HMP-1 and HBP both play a role in host cell activation by *H. pylori*. Thus, we have produced solid evidence to challenge the previous assumption that ADP-heptose is the sole immune-active metabolite produced by *H. pylori* and other bacteria.

4.1.3 Factors for strain-specific variation of heptose activation

Due to the sequence variability of the central heptose biosynthesis enzyme HldE between strains, especially in contrast to the other heptose biosynthesis enzymes that are highly conserved (Stein, Faber et al. 2017 and Publication II), and its bifunctional activity due to the possession of two functional distinct domains, we had a particular interest in HldE, its expression and enzymatic activity. HldE is crucial for the heptose-dependent pro-inflammatory activation of host cells in different species (Gaudet, Sintsova et al. 2015, Milivojevic, Dangeard et al. 2017, Zhou, She et al. 2018) including *H. pylori* (Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). These previous findings are supported by this work. In Publication II and our previous studies (Olbermann, Josenhans et al. 2010, Stein, Faber et al. 2017) we show strain variability on transcript and protein level, which is one likely explanation for the strain-specific differences in cell activation of live bacteria and lysates. Using HldE of different *H. pylori* strains for the *in vitro* synthesis of heptose metabolites, we observed strain-specific differences resulting of active metabolites. The variability in enzymatic activity of HldE, the variable sequence, transcript and expression data, demonstrate an important role for HldE to the strain-specific outcomes of the heptose biosynthesis. Mass spectrometry analysis of the *in vitro* products synthesized by GmhA and HldE, as well as using the first domain of HldE instead of the full enzyme (HldE_d1), revealed high amounts of HMP-1. To produce HMP-1 from HBP, a phosphatase is required, a step in the synthesis cascade which is supposedly carried out by GmhB (Kneidinger, Marolda et al. 2002). The fact that we actually find HMP-1 even in the absence of GmhB, strongly suggests a further enzymatic function of HldE, possibly a phosphatase activity. Additional activity of HldE could shed light on the controversy of whether or not GmhB is an essential enzyme for heptose-dependent signaling. Our study indicates that a *gmhB*-defective mutant produces HBP and HMP-1, since activity potential of mutant lysate

strongly increased upon transfection compared to the parental strain, suggesting activity from a permeable metabolite other than ADP-heptose, very likely HMP-1, together with a non-permeable metabolite, probably HBP. However, further studies examining the functional activity of HldE are needed to strengthen this hypothesis. In addition, we find other factors for strain-specific heptose dependent activation, too. In addition to the variability of HldE, we were also able to show variability in the transcripts of the other heptose biosynthesis genes. This suggests that the pathway enzymes may not be expressed to the same level in all the strains. This might ultimately lead to the production of different metabolites in a strain-specific and variable composition of the overall heptose metabolite production. This is supported by our work, as we show, when comparing different *H. pylori* wild type strains, that transfection of the different lysates does not enhance activity to the same extent (Publication II). The lysates are likely to contain different compositions of permeable and non-permeable metabolites. In total, the observed strain-specific differences in heptose dependent activation seem, according to our data, to be the result of strain-specific enzyme activities, strain-specific regulated transcript amounts of the heptose biosynthesis gene cluster and variable metabolite compositions.

4.2 Control of heptose metabolites production in the context of global bacterial regulation

The human stomach is often described as hostile environment and was for a long time even considered to be sterile before *H. pylori* was discovered (Warren and Marshall 1983, Yang, Nell et al. 2013). To persistently colonize this habitat, *H. pylori* needs to be highly adapted and flexible, responding to changing conditions. Although *H. pylori* is able to sense stimuli from its environment, the pathogen has been found to lack some classical global regulators known from other bacteria (Skouloubris, Thiberge et al. 1998, Baker, Raudonikiene et al. 2001, Bijlsma, Waidner et al. 2002). In our work, we show that some of these environmental factors are involved in the overall variability of heptose-dependent signaling in *H. pylori* (Publication II). Variation in quantity and quality of produced metabolites and differences in heptose biosynthesis and regulation even between different strains and under certain environmental conditions need a complex and fine-tuned regulation. In Publication II we obtained some evidence that the regulatory protein CsrA is involved in the regulation of heptose (metabolic) activity and metabolite production. In doing so, we contribute to the knowledge of alternative regulatory mechanisms in *H. pylori* with a focus on the production of heptose metabolites, but also global regulation, as an important factor to govern the decision making between alternative lifestyles during the interaction of the pathogen with its host.

4.2.1 The post-transcriptional regulator CsrA is involved in the regulation of heptose metabolite biosynthesis

Our data suggest that, carbon storage regulator A (CsrA) is involved in regulation of the heptose gene cluster in *H. pylori*. CsrA is a posttranscriptional regulator, canonically working in cooperation with small RNAs, that was originally described as potent repressor of glycogen synthesis in *E. coli* (Romeo, Gong et al. 1993). At this time, the full spectrum of regulatory roles of CsrA could only be guessed at (Romeo 1998). To date, CsrA is known to coordinate the global regulation of important processes such as carbon metabolism and motility, but also virulence in various bacteria, reviewed by Vakulskas et al. (Vakulskas, Potts et al. 2015). CsrA is also of regulatory importance in *H. pylori*, as it contributes to the regulation of target transcripts in stress responses and virulence (Barnard, Loughlin et al. 2004). Further, a study by Kao et al. investigated the global CsrA involvement in *H. pylori* using a comparative transcriptomic study (Kao, Chen et al. 2017). Prior investigation of flagella formation and expression of major flagellins in a CsrA-defective mutant already suggested that CsrA of *H. pylori* controls bacterial motility by regulating flagella formation, in strain J99 (Kao, Sheu et al. 2014). Looking at the comprehensive RNA-seq analysis, Kao et al. found 53 differentially expressed genes comparing a CsrA mutant with its parental strain, showing a broad impact of CsrA on a variety of target genes in *H. pylori* with a focus mainly on flagella formation and motility (Kao, Chen et al. 2017). Despite this study being important for our work as it seems to connect metabolism, motility and sessility regulation, the connection between CsrA and heptose regulation has not yet been explored before in *H. pylori* or any other bacterium. In *B. subtilis* and a close relative of *H. pylori*, *C. jejuni*, a small CsrA-binding protein, FliW, has been implicated in the function of CsrA for motility regulation (Dugar, Svensson et al. 2016, Radomska, Wösten et al. 2017, Altegoer, Mukherjee et al. 2018). A *fliW*-like gene is annotated in various *H. pylori* genomes, however its function and possible connection with motility and/or CsrA regulation has not been explored yet. Investigating the influence of certain environmental factors, in the present thesis, we also looked at the regulation of the heptose gene cluster in the presence or absence of an active T4SS, knowing that heptose transport and signaling in the human target cells depends on the transport system (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). Since an active T4SS indeed plays a role in heptose cluster gene expression (Publication II), a comprehensive transcriptome analysis was performed to investigate the effects of an absent secretion system on global gene regulation. The results showed some overlap with previously identified CsrA-regulated transcripts as reported by Kao et al. (Kao, Chen et al. 2017), providing preliminary evidence in our work that heptose-dependent regulation is

under control of the regulatory protein. Interestingly, the transcripts of heptose biosynthesis genes were also upregulated in the presence of human gastric epithelial cells. The presence of the cells also affected the *csrA* transcript itself (feedback loop) and genes controlled by CsrA, suggesting a CsrA-dependent feedback regulation of heptose production upon cell contact. CsrA-deficient mutants provided further evidence, as heptose transcript and heptose-dependent pro-inflammatory signaling were impaired when comparing mutants with the parental strain (Publication II). Thus, we conclude that CsrA is involved in the regulation of heptose biosynthesis and therefore in heptose-dependent activation of host cells. From the comprehensive transcriptomics work in *E. coli* by Potts et al., it is known that CsrA can have various roles and activate and repress several targets at the same time (Potts, Vakulskas et al. 2017). Further, CsrA can act as regulatory protein via different mechanisms, impacting translation, RNA abundance and RNA stability of a variety of target genes (Potts, Vakulskas et al. 2017). Given the extensive regulatory functions of CsrA (Kao, Chen et al. 2017, Potts, Vakulskas et al. 2017), this could also have global implications for *H. pylori* infection and interaction with the host that are certainly worth investigating further.

4.2.2 CsrA controls lifestyle and virulence in *H. pylori* and other bacteria

In its niche, *H. pylori* can be found attached to the epithelial cell layer but also alternatively swimming through the mucus (Yang, Nell et al. 2013). Upregulation of the heptose gene cluster upon contact between *H. pylori* and epithelial cells (Publication II) points to an upon-demand production of heptose metabolites. The fact that *H. pylori* appears to produce more metabolites in the presence of cells may indicate that heptose production may be a lifestyle decision in which *H. pylori* produces more metabolites in a sessile stage near epithelial cells than in a planktonic, free swimming stage. Such a switch is also known from other bacteria like the enterobacteria *S.* Typhimurium (Jonas, Edwards et al. 2010) and *E. coli* (Jackson, Suzuki et al. 2002, Elbaz, Socol et al. 2019) as well as from *Pseudomonas* species (Mikkelsen, Sivaneson et al. 2011, Sadiq, Flint et al. 2017, Thompson, Hall et al. 2023). This allows a choice of lifestyle that is highly adapted to the respective niches. Considering the use of heptose metabolites for the interaction with host cells, a controlled switch to production of heptoses exceeding the amount needed for the mere LPS house-keeping function, may also have another benefit for *H. pylori*. Production of heptose metabolites on demand to interact with the host may conserve resources and energy. Such strong lifestyle switches are important for the survival of all bacteria in their alternative environments and have already been shown for various pathogens. For *S.* Typhimurium, adaptation to different niches in the host is critical for bacterial survival and requires switching be-

tween different lifestyles. Jonas et al. demonstrated how CsrA controls motility and biofilm factors via a c-di-GMP specific phosphodiesterase and *fliA*, thereby orchestrating the motility-sessility switch in *S.* Typhimurium (Jonas, Edwards et al. 2010). Similarly, *P. aeruginosa* can switch lifestyles via the c-di-GMP and Gac/Rsm signaling network. Several regulatory systems control the lifestyle shift and enable adaptation to environmental changes, reviewed by Mikkelsen et al (Mikkelsen, Sivaneson et al. 2011). One important control system for the switch from sessile to motile is the Rsm system (repressor of secondary metabolism), which includes RsmA, a CsrA homolog (Mikkelsen, Sivaneson et al. 2011, Pourciau, Lai et al. 2020). In *E. coli*, CsrA has been shown to control biofilm formation which has also been discussed to be involved the decision between sessile and planktonic state (Jackson, Suzuki et al. 2002). Further, it was shown that CsrA, together with other factors, is involved in the alternation between cell-attached and planktonic state of enteropathogenic *E. coli* (EHEC), also involving the regulation of its T3SS (Elbaz, Socol et al. 2019). It was also shown for *Y. pseudotuberculosis* (LeGrand, Petersen et al. 2015) and *Y. enterocolitica* (Kusmierek, Hoßmann et al. 2019) that CsrA is a factor of T3SS regulation. These examples demonstrate the universal importance of CsrA for adaptive lifestyle change. Our data provide evidence for such a switch under the control of CsrA in *H. pylori* and adds initial insight to this research field, as there is currently limited information on CsrA and potential lifestyle switching (Barnard, Loughlin et al. 2004, Kao, Sheu et al. 2014). In the intestinal pathogen *S.* Typhimurium, CsrA regulates genes necessary for gastrointestinal mucosal invasion (Altier, Suyemoto et al. 2000), suggesting that CsrA may also be involved here in modulating host-pathogen interactions. In *P. aeruginosa*, RsmA is not only implicated in the switch from motile to non-motile, but also contributes to the interaction with the host. It has been found that RsmA positively regulates the T3SS, thereby allowing direct interaction with human airway epithelial cells (Mulcahy, O'Callaghan et al. 2006). This positive regulation of the T3SS is a virulence trait associated with acute infection. In chronic disease, the acute infection reoccurs which can have serious health consequences for the patients. RsmA has been shown to control this switch by inversely regulating certain virulence factors associated either with the acute infection, as for T3SS and motility, or with the chronic disease, such as factors involved in biofilm formation (Mulcahy, O'Callaghan et al. 2008). Thus, the pathogen serves as a good example of how RsmA (CsrA) regulates virulence lifestyles. A *P. aeruginosa rsmA* mutation has also been associated with a decreased ability to initially colonize mice, however ultimately leading to increased persistent infection (Mulcahy, O'Callaghan et al. 2008). Similarly, this was also shown for *H. pylori*, as CsrA-deficient strains were attenuated in virulence in the early mouse infection model (Barnard, Loughlin et al. 2004). In

addition, Bernard and colleagues found further evidence for the role of CsrA in regulating *H. pylori* physiology through environmental stimuli which may facilitate adaptation to varying environmental conditions during colonization (Barnard, Loughlin et al. 2004). It is further known for *V. cholerae*, that CsrA promotes survival and adaptation, has a central role in virulence regulation, and therefore fosters intestinal colonization (Butz, Mey et al. 2021). Based on our results and the role of CsrA (RsmA) in other bacteria (Lucchetti-Miganeh, Burrowes et al. 2008), it does not seem far-fetched that CsrA allows *H. pylori* to switch between a less virulent, motile state and a sessile state with higher virulence during chronic infection due to balanced production of heptose metabolites, but also through feedback by intracellular heptose concentration. The heptose balance and CsrA regulation may then also affect the functionality of the secretion system, similar to CsrA (or its homologe RsmA) being involved in regulation of the secretion system in *P. aeruginosa* (Mulcahy, O'Callaghan et al. 2006), EHEC (Elbaz, Socol et al. 2019, Zacharia, Pal et al. 2022) and *Yersiniae* (LeGrand, Petersen et al. 2015, Kusmierek, Hoßmann et al. 2019) thus contributing to a niche-specific lifestyle.

4.3 Advantage of heptose-dependent inflammation in infection

Heptose metabolites classify well as MAMPs (Medzhitov 2007) as they are produced by most Gram-negative bacteria but are absent from the human glycome (Herget, Toukach et al. 2008). Moreover, there is a strong selective pressure on the synthesis of heptoses, as heptose-devoid mutants produce a deep-rough LPS phenotype (Provost, Harel et al. 2003, Yu, Wang et al. 2016). This makes the mutants more susceptible to environmental stresses, resulting in reduced virulence in animal models (Valvano, Messner et al. 2002, Seregina, Petrushanko et al. 2022). *H. pylori* heptose-less mutants show loss or strongly reduced activation on epithelial cells as well as myeloid cells, as demonstrated in both Publications I and II and in previous work of our group and others (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017). Heptose-deficient mutants of other species, *S. flexneri* (Milivojevic, Dangeard et al. 2017), *Y. pseudotuberculosis* (Zhou, She et al. 2018) and *C. jejuni* (Cui, Duizer et al. 2021) resulted in a similar phenotype. This shows that activation of host cells by heptoses accounts for a large portion of innate immune activation by various species, which raises an important question: What is the benefit of inducing inflammation, by heptose metabolites?

4.3.1 The variation in quantity and composition of heptose metabolites as a tool for balanced host modulation in *H. pylori* infection

H. pylori lives in an unstable environment and faces changes in its environment requiring frequent genetic adaptation and the interaction with its host (Suerbaum and Josenhans 2007). Flexibility in host modulation by the use of different metabolites, however, may add another layer to the high adaptability of *H. pylori*. As discussed in 4.1.2, some metabolites may cause an immune reaction of host cells that is delayed or less efficient, thus providing a tool to influence the strength of cellular activation to balance inflammation and persistent colonization. The strength of immune reaction might be influenced by two different set screws, the quantity and the composition of heptose metabolites. Based on our results obtained in Publication II, it seems possible that *H. pylori* elicits immune activation in a dose-dependent manner, by producing (or secreting) more or less of the metabolite. *P. aeruginosa*, one of the most prevalent bacterial colonizers in cystic fibrosis patients, can regulate inflammation by producing a certain amount of quorum sensing molecules. A small amount of molecules elicits an immune response, whereas a high concentration has inhibitory effects (Souche, Vandenesch et al. 2023). This example shows, that modulation of the host immune response is possible by controlling the strength of the immune response caused by bacterial effectors, such as the quorum sensing molecules or in our study, possibly the heptose metabolites. Our results indicate that inflammation is triggered to different extents, which we show using various *H. pylori* strains, different metabolites, and dilution series of three metabolites. Therefore, our findings contribute to the hypothesis that *H. pylori* might use heptose metabolites to fine-tune the strength of the inflammation to keep up a good balance in its niche (see 4.3.3). Further, it was shown, that *H. pylori* evolves very distinctly depending on the micro-location in the stomach (Ailloud, Didelot et al. 2019) raising the question whether heptose-dependent signaling is also regulated differently depending on the location of the infection site in the stomach. Upregulation of heptose biosynthesis enzymes in close cell-contact indicates different production in presence or absence of the cells, suggesting different heptose output in areas where *H. pylori* has more cell contact, close to the epithelial layers, compared to a planktonic state. The different heptose output however does not necessarily have to result from enhanced production of metabolites, also a change in composition of different metabolites is possible. Supporting this hypothesis, we show that different metabolites trigger the immune response to a different extent (Publication II). In *K. pneumoniae* infection, it was shown that the fitness cost of *gmhB* deletion was different depending on the infection site and pathogenesis phase. Whereas heptose biosynthesis enzyme GmhB was required for bloodstream survival, it was not required for lung inflammation by *K. pneumoniae* indi-

cating that ADP-heptose may not be required (Holmes, Smith et al. 2022). This may be a hint to the use of different metabolites to elicit niche-specific immune activation, which is, seen our data, also conceivable for *H. pylori* infection. One of the environmental factors influencing the heptose production may be the presence of the CagT4SS, since the heptose operon was less expressed in absence of the transport system (Publication II). As we know from previous studies (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017) and the two Publications, the heptose signaling is dependent on the Cag secretion system. This is also particularly interesting when discussing the benefit of producing different metabolites, as these do not all display the same properties. In contrast to ADP-heptose and HMP-1, HBP was shown to be non-permeable to the host cell membrane (Zhou, She et al. 2018), underlining the need of a secretion system for the activation of the host cells by HBP. Thus, it is also conceivable that *H. pylori* may produce different metabolites depending on the presence/absence or activity of the secretion system among other factors. This points to a very complex interplay between the use of different metabolites, the role of the T4SS and the close surrounding of *H. pylori*. Together, this creates an important tool for *H. pylori* to control host responses and thereby ensuring an environment sufficient for persistent colonization.

4.3.2 Transport of heptoses through a secretion system: advantage or disadvantage in heptose-dependent inflammation?

Heptoses have been shown to be released into the bacterial supernatant by *N. meningitidis* (Malott, Keller et al. 2013, Gaudet, Sintsova et al. 2015) and *C. jejuni* (Cui, Duiz*er et al. 2021)*. Therefore, it could be assumed that the presence of a secretion system does not seem to be a general prerequisite for immune activation by the MAMPs. On the other hand, *H. pylori* (Gall, Gaudet et al. 2017, Stein, Faber et al. 2017, Zimmermann, Pfannkuch et al. 2017) and *Y. pseudotuberculosis* (Zhou, She et al. 2018) were shown to rely on their secretion system to induce pro-inflammatory activation via heptose signaling. In Publication I we further showed that the activation potential of *H. pylori* supernatants was extremely low compared to bacterial lysates, suggesting that the gastric pathogen does not readily release metabolites in its surrounding. Since some bacteria manage to cause heptose-dependent inflammation without possessing a secretion system, the important question arises as to why other bacteria use their secretion system for the same purpose. The results obtained in this work can contribute to the discussion of this question, as we show that *H. pylori* uses different heptose metabolites, ADP-heptose, HMP-1, and HBP, three metabolites with different permeability (Publication II). Our data implies that host cell activation by HBP depends

on active transport, suggesting that the use of a secretion system may be advantageous for *H. pylori* to be more flexible in the use of different metabolites to stimulate its host (see also 4.3.1). Whether this may also be the case with other bacteria remains to be explored. Adding our recent findings, the decrease in gene transcript encoding heptose synthesis enzymes in T4SS mutants, it appears that the T4SS also plays a role overall in regulating heptose biosynthesis. Thus, the secretion system of *H. pylori*, and possibly of other heptose-producing pathogens that have a secretion system, may be important not only for active transport but also for fine-tuning the production of specific metabolites and thus for the overall balance in inflammation. Considering other *H. pylori* effectors that are directed to host cells by the CagT4SS to activate them, it also seems beneficial to direct effectors to host cells for efficient activation. But then how do pathogens manage to cause heptose-dependent activation without a transport system? Interestingly, the lifestyle of *C. jejuni* and *N. meningiditis* provides a possible explanation. As *C. jejuni* can survive in intestinal host cells after invading them (Watson and Galán 2008), active transport inside the host cell may not be needed. Further, *N. meningiditis*, despite being classified as extracellular pathogen, also has a facultative intracellular lifestyle (Dumenil 2011). Thus, in this state, metabolites would not be needed to be actively transported either. With a changing lifestyle from extracellular to intracellular and the ability of both the pathogens to produce different permeable and non-permeable metabolites, as suggested by transfection results, active transport of metabolites through secretion systems may not be required. Depending on the purpose of the transport, possessing a secretion system may not only be unnecessary but even a disadvantage. Directed transport may enhance the specificity but on the other hand, it limits the distribution of effector molecules to a specific location and quantity. Undirected release may therefore be the better choice for a rather unspecific distribution of high amounts of effectors. This shows that the need for a secretion system absolutely depends on the type and amount of effectors, as well as on the purpose of the transport.

4.3.3 General benefit of heptose-dependent signaling in acute and chronic infection

Heptose-dependent signaling has been demonstrated for several pathogens leading to both an acute but also chronic infection. An example of a pathogen that produces heptose during acute infection is *S. flexneri*, which causes acute intestinal inflammation in its human host (Milivojevic, Dangeard et al. 2017, García-Weber, Dangeard et al. 2018). Although the invasive pathogen has evolved a number of tools to downregulate the immune response of infected cells (Kim, Lenzen et al. 2005, Yang, Hung et al.

2015), heptose-dependent signaling via the TIFA-ALPK1 route was shown to be essential for IL-8 induction (Milivojevic, Dangeard et al. 2017). Similarly, in the same work, *S.* Typhimurium and *N. meningitidis* and by Cui and colleagues *C. jejuni*, all causes of acute infections in humans, were demonstrated to evoke immune responses through heptose signaling (Milivojevic, Dangeard et al. 2017, Cui, Duizer et al. 2021). A prominent example for causing chronic infection is the model organism of this thesis, *H. pylori*. As a rather immune evasive organism colonizing its host lifelong if not treated, *H. pylori* hampers clearance of the infection by the immune system. The heptose-dependent signaling resulting in strong immune activation seems to be in contrast to that, especially since signaling was also shown to harm the host cells by causing DNA damage (Bauer, Nascakova et al. 2020) which was even discussed to potentially contribute to the development of gastric cancer in *H. pylori* infection (Duizer and de Zoete 2023). Since the signaling has consequences for the host, in which *H. pylori* lives for decades, inflammation triggered by heptose metabolites also must have benefits for *H. pylori*. In support of this, *H. pylori* has been shown to have evolved around the globe, resulting in large geographic differences. During migratory evolution, its pro-inflammatory effect seems to have increased. An example are the East Asian strains, that underwent evolutionary bottle necks and are highly virulent (Yuan, Yan et al. 2017). This suggests that increased inflammation was favored during the evolution of *H. pylori*. Since heptoses also have a housekeeping function, the question remains to be answered as to when heptoses evolved to also be used to trigger the immune system rather than solely being incorporated into the LPS. Further, it was discussed for the *H. pylori* infection before, that inflammation might contribute to the nutrient availability as *H. pylori* was shown to cause damage of the epithelial barrier (Wroblewski and Peek 2011). This leads to the release of amino acids that *H. pylori* may be able to metabolize, which is an important factor for colonization. However, this hardly seems to be the only cause of heptose-dependent inflammation. Other bacteria which can cause a chronic infection were found to produce active heptose metabolites, including *Y. pseudotuberculosis* (Zhou, She et al. 2018) and *K. pneumonia* (Holmes, Smith et al. 2022). Nonetheless, the diversity of bacteria that produce heptose metabolites to elicit an immune response demonstrates the universal importance of heptose in infection, regardless of acute or chronic lifestyle. Most of the persistently colonizing pathogens exhibit immune-avoidance properties, raising the question of why these bacteria use heptose-dependent signaling to provoke an immune response when, on the other hand, they attempt to evade the immune system. When looking at heptose from a more universal aspect, examining not only pathogens but also commensals, Martin-Gallausiaux and colleagues came to an interesting conclusion. Considering the proper-

ties of heptoses as MAMPs, these microbial patterns are generally found not only in pathogenic bacteria but also in microorganisms of the commensal microbiota. *Akkermansia muciniphila* was found to activate its host cells via the same heptose-dependent pathway (Martin-Gallausiaux, Garcia-Weber et al. 2022), despite being known as commensal with positive effects on the human health (Everard, Belzer et al. 2013). This is a particularly important finding, as it shows that heptose-dependent inflammation by *A. muciniphila* contributes to a balanced homeostasis in the gut. However, which heptose metabolites the commensal produces and in which quantities needs to be further elucidated. Still, the use of heptose metabolites to balance colonization and inflammation appears to be a useful tool not only for non-infectious commensals, but for any pathogen that relies on the stability of its host for colonization. Thus, heptose-dependent signaling may play an important role for many pathogens to modulate immune response of their hosts leading to a balanced environment which may also depend on the type or amount of metabolites used. For *N. gonorrhoeae*, it has been cautiously speculated that innate activation by heptose metabolites may contribute to a balanced lifestyle between asymptomatic colonization and inflammatory disease (Malott, Keller et al. 2013). In support of this hypothesis, there is data of different studies summarized by Garcia-Weber and Arrieumerlou (2021) which indicate that ALPK1 is playing a role in promoting intestinal homeostasis. Heptose metabolites as a tool to balance inflammation levels appear to be beneficial, as heptose-dependent signaling facilitates cross-talk between pathogen, host cells and the host immune system.

5. Outlook

The work presented and discussed in this thesis has helped to advance the knowledge of heptose-dependent signaling by *H. pylori* to different types of host cells. Overall, our results helped to elucidate novel mechanisms of *H. pylori* to modulate host interactions through the fine-tuned production of bacterial effector metabolites. However, our results have raised further questions worthy of future investigation.

In Publication I, we show heptose-dependent activation of macrophage-like cells and primary macrophages. Together with our additional study in neutrophils, this demonstrates that *H. pylori* indeed elicits similar responses with heptose metabolites in various types of host cell types, leading to the activation of NF-*κ*B and the secretion of cytokines at early infection timepoints. However, how heptose-dependent signaling influences myeloid cells in disease *in vivo*, still remains unclear. In addition, we investigated the activation of phagocytic cells, macrophages (Publication I) and neutrophils (Faass, Hauke et al. 2023 B). However, the heptose-dependent activation of other antigen presenting cells such as dendritic cells remains unexplored. In dendritic cells, other heptose-related *H. pylori* factors such as the role of the T4SS in *H. pylori*-induced activation have been investigated so far (Kim, Park et al. 2013, Neuper, Frauenlob et al. 2020, Faass, Hauke et al. 2023 A) and also need to be investigated for their response upon stimulation with different heptose metabolites.

Further biochemical work is required to comprehensively map the identity of heptose metabolites in diverse bacterial lysates and to improve detection of the various metabolites in bacterial and *in vivo* samples. Also, the CsrA-related regulatory phenomena such as decreased transcription of the heptose biosynthesis operon and the lower activation potential of CsrA-defective mutants require biochemical analysis of heptose metabolites in quantity and composition in absence of the regulator or under various conditions. Difficulties in detecting metabolites in lysates and further studies should benefit from a technical advance here. It will be worthwhile to find ways to lower the detection limits for certain metabolites, artificially increase the amount of metabolites, or facilitate identification by labeling.

As discussed above, different metabolites have different properties, e.g. concerning cell permeability, which also has implications for the possible transport mechanisms. Despite the established knowledge that heptose signaling depends on an active CagT4SS in *H. pylori* and the influence of the CagPAI on heptose biosynthesis (Publication II), it is still unclear how these metabolites are transported. Which proteins of the

CagT4SS apparatus are involved and how is the process regulated? Since the heptose biosynthesis gene expression is enhanced upon cell contact, it can be speculated that also transport of metabolites is enhanced or even activated upon cell contact. This also concerns a global regulatory switch between different lifestyles, host cell associated (sessile) or motile.

Taken together, the interplay between pathogen, host, immune system and metabolism appears to be beneficial for *H. pylori* infection, but open questions remain. On the one hand, the regulation of heptose biosynthesis needs further attention, above all since we found CsrA to be involved in the control of the heptose production, probably in response to environmental changes. But we still lack knowledge how exactly CsrA regulates. CsrA can act as regulatory protein via different mechanism, impacting translation, RNA abundance and RNA stability of a variety of target genes (Vakulskas, Potts et al. 2015, Potts, Vakulskas et al. 2017). On the large scale of different functions, other factors (proteins and RNAs) which are involved in the complex regulatory mechanism and therefore in a potential lifestyle switch of *H. pylori* need to be identified in the future, including comprehensive analysis of CsrA-deficient *H. pylori* strains. In addition, further looking into CsrA-function in the context of heptose signaling, other aspects, like influences on bacterial metabolism or motility should also be taken into account. Preliminary data (not shown) indicated that the bacterial metabolism undergoes changes upon feeding of bacteria with ADP-heptose, underlining the need to perform studies on metabolism in the context of heptose production and regulation. These aspects may deserve a closer look also in a strain-specific manner as we and others have shown, that regulation is strain-specific in *H. pylori*. On the other hand, interaction always requires two (or more) partners, which is why a closer look onto uptake mechanisms and cellular changes in response to stimulation with heptose metabolites is an important aspect, too. Although the detection pathway is established, questions about ALPK1-binding of alternative metabolites or the change in host metabolism upon heptose-activation remain. Consequences of immune-metabolic changes in host cells for the host-pathogen interaction or the discussed cross-talk need further attention in future projects.

An overall goal of *H. pylori* research is to find new perspectives for treatment or prevention of the infection as it can have various severe outcomes (Suerbaum and Michetti 2002, Malfertheiner, Camargo et al. 2023). Since ADP-heptose very efficiently leads to pro-inflammatory activation in its host cells causing inflammation of the stomach tissue, the search for potential inhibitors may be an interesting research branch and a putative novel antimicrobial strategy in *H. pylori*. Given the universal role of heptose metabolites in various different species, this field may certainly be worth considering for other pathogens as well.

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List of other publications (not part of thesis)

Publications:

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