O-Antigen Protects Gram-Negative Bacteria from Histone Killing

Catherine Chaput1,a, Eileen Spindler2,b, Ryan T. Gill2, Arturo Zychlinsky1,*

1 Department of Cellular Microbiology, Max-Planck Institute for Infection Biology, Berlin, Germany, 2 Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado, United States of America

Abstract

Beyond their traditional role of wrapping DNA, histones display antibacterial activity to Gram-negative and -positive bacteria. To identify bacterial components that allow survival to a histone challenge, we selected resistant bacteria from homologous Escherichia coli libraries that harbor plasmids carrying pieces of the chromosome in different sizes. We identified genes required for exopolysaccharide production and for the synthesis of the polysaccharide domain of the lipopolysaccharide, called O-antigen. Indeed, O-antigen and exopolysaccharide conferred further resistance to histones. Notably, O-antigen also conferred resistance to histones in the pathogens Shigella flexneri and Klebsiella pneumoniae.

Introduction

Natural antimicrobial peptides (AMPs) are thought to regulate both the symbiotic flora on the mucosa and immune defense, for example through the recruitment of inflammatory cells [1–3]. AMPs are stored in lysosomes and granules of macrophages and neutrophils respectively. Defensins, for example, are often secreted by epithelial cells. Interestingly, histones are one of the most potent antimicrobials isolated from fish and toads [4–11]. In eukaryotes containing Km 100 g/ml, CaCl2 10 mM and MNase 40 U/ml

Materials and Methods

Bacterial Strains and Growth Conditions

We used the E. coli strains MACH1-T1® (referred to as MACH1) and TOP10 (from Invitrogen), S. flexneri strains M90T and the isogenic waaL and rfbA mutants (Manuscript in preparation: Geldmacher et al.), K. pneumoniae strains, wild type 52143, the unencapsulated (52K10) or O-antigen- (52O21) mutants, were kindly obtained from Regis Tournebize and Jose A. Bengoechea [16]. Bacteria were grown in Lysogeny Broth (LB) media, except for S. flexneri, which was grown in TSB. Medium was supplemented, when necessary, with kanamycin (50 μg/ml, Km), chloramphenicol (50 μg/ml, Cm) and/or L-arabinose. Histones from calf thymus (Calbiochem, H9250) were used as provided by from calf thymus (Calbiochem, H9250) were used as provided by

Selection Procedure

The E. coli libraries consisted of MACH1 pSMART-LCKm carrying random fragments of 1, 2, 4 and 8 kb of E. coli genome as previously described [17]. A pool of the 4 libraries was selected in 2 steps repeated 3 times. In the first step, the libraries (adjusted to an OD 600 nm of 0.1) were treated with 25 μg/ml of histones during 3 h at 37°C with agitation in HAH medium (HBSS+, casamino acid 0.9% and HEPES 10 mM) containing Km. In the second, the recovery, step, we added one volume of LB 2X containing Km 100 μg/ml, CaCl2 10 mM and MNase 40 U/ml

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* E-mail: catherine.chaput@charite.de (CC); zychlinsky@mpiib-berlin.mpg.de (AZ)

a Current address: Department of Infectious Diseases and Pulmonary Medicine, Charité University Hospital, Berlin, Germany

b Current address: Opxbio, Boulder, Colorado, United States of America
(Finnzymes) with the primers mention in Table 1. The PCR products were digested with restriction enzymes (from Fermentas) according to the restriction site present on the primers. The digested PCR product was inserted by ligation (T4 DNA Ligase from Fermentas) in pBAD18 (KmR) and pBAD33 (CmR) vectors digested with the same restriction enzymes. The ligation products were introduced into E. coli TOP10 by heat shock transformation (Invitrogen) and selection was carried on LB agar with the appropriate antibiotic. After checking the inserts by sequencing, the plasmids were introduced into MACH1 for further analysis. The clones obtained are summarized in Table 2.

Histone killing Assay

For MACH1 carrying pBAD33 and/or pBAD18 constructs were grown overnight in LB with antibiotics and L-arabinose 0.4%. S. flexneri and K. pneumoniae were grown in TSB and LB respectively for 3 h prior to the assay. Bacteria were harvested and resuspended in HAH medium. The bacterial suspensions were adjusted to a final concentration of 10^9 bacteria/ml and co-incubated with different histone concentrations. The histone concentrations tested started from 100 μg/ml, or 250 μg/ml for K. pneumoniae, down to 0.625 μg/ml by serial dilution of 1/2. The surviving bacteria were enumerated by serial dilution in PBS MgCl₂ 1 mM and plating. The bacterial enumeration determined the minimal bactericidal concentration of histones to kill 90% (MBC90) and 99% (MBC99) of the inoculum after 1 h of incubation.

LPS Crude Extract Analysis

LPS was extracted from plate cultures by the proteinase K method [18]. LPS samples were separated by Tris-Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (gradient gel 10–20%, Criterion Biorad) as described by Lesse and colleagues [19]. The LPS was visualized by silver staining after periodate treatment [20].

Exopolysaccharide Quantification

The exopolysaccharides of E. coli over-expressing the selected genes were extracted and purified from overnight liquid culture containing arabinose as previously described [21]. The culture was first boiled and after spinning down the bacterial remnants, the supernatant was precipitated with ethanol and then dialyzed. To quantify the concentration of exopolysaccharides, we determined the carbohydrate concentration (mg/ml) with the glycoprotein carbohydrate estimation kit (Thermoscientific).

Results

Positive Selection of Libraries of Histone Resistant E. coli

To determine mechanisms responsible for histone resistance, we set up a screen based on positive selection of E. coli libraries, which over-express random genomic fragments [17]. We used a pool of four libraries consisting of E. coli carrying plasmid harboring random fragments of the E. coli K12 genome (Fig. 1A). Each library covers the entire E. coli genome in 1, 2, 4 and 8 kb fragments allowing us to screen the genome several times and, depending on the size of the fragment, refine the region conferring the expected phenotype [17]. Thus, the selection was designed to enrich histone resistant clones. Because in vivo bacteria encounter more than one kind of histone, we used a mixture of histones isolated from calf thymus. Prior to the selection, we tested the antimicrobial activity of the whole histones as provided by the manufacturer or after re-purification by chromatography. The re-purification step did not change the dose or kinetics of E. coli killing.
Thus, the positive selection of the pooled libraries was confirmed by: (i) the increasing bacterial concentration of the libraries after incubating them with histones, (ii) the increase in histone resistance of clones isolated from the libraries, and (iii) the enrichment of one particular plasmid insert in clones isolated from the selected libraries.

We were interested both in the most abundant as well as in rarer fragments that contribute to histone resistance. Hence, we compared the plasmid population from the initial and selected pooled libraries by microarray analysis. As a control, we confirmed that there was no selection in the sample obtained from control libraries that were not exposed to histones through the experiment.

In contrast, 8 kb fragments carrying the woeJ, cpsB, cpsG and woeA genes were strongly enriched after histone selection. This data is in agreement with the sequencing data presented in Fig. 1C.

The genes present in the woeJ/I fragment belong to the colanic acid cluster. Interestingly, in the microarray analysis we also found the wzc/wcaA fragment, which also belong to the colanic acid cluster, albeit at lower intensity (Fig. 1D). The intensity of a spot in microarray was normalized by the logarithmic value of the intensity of the spot corresponding to the DNA fragment divided by the logarithmic value of the intensity for the vector’s backbone (see Materials and Methods). Then, the fitness attributed to a spot or gene was calculated by comparing the intensities before and after selection. Overall data of the fitness are in Table S1. On average, the woeJ/I fragment had fitness of 9.1 in comparison with 2.1 for the wzc/wcaA fragment (Fig. 1D).

### Cloning Under Arabinose Promoter and Toxicity of Over-expressed Genes

The colanic acid cluster encodes genes required for the production of exopolysaccharide and for semi-smooth LPS form [21,22]. Intriguingly, we did not select all the genes of the cluster (Fig. 2A). Instead, we selected for enzymes involved in, (1) the synthesis of sugar precursor in the cytoplasm, CpsB and -G, (2) the assembly of the sugar unit at the inner membrane by the glycosyltransferases (cytoplasmic side), WcaJ, -I and –A, and (3) the export of the polysaccharide, Wzc.

To determine which genes in this cluster were required to confer histone resistance, we cloned all the genes in vectors either

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**Table 1.** Primers designed for the different clonings of the wcal/J and wzc/wcaA fragments in pBAD33 and pBAD18 (see Fig. 2).

| Name   | Sequence                                                                 | Restriction |
|--------|--------------------------------------------------------------------------|-------------|
| wcal_F02 | GGGTACCCCGgaggagAAACGATG AAAATACGCTTAGCCGAC                       | KpnI        |
| wcal_R01 | AACTGCAGTTTTACCGGAAATATCTTTATATATTG                                 | PstI        |
| cpsB_F02 | GGGTACCCCGgaggagAAACGATG GGGCAGTGAACACTTACCC                       | KpnI        |
| cpsB_R01 | AACTGCAGTTTACCGGCAATTCGAGCTACCC                                           | PstI        |
| cpsG_F02 | GGGTACCCCGgaggagAAACGATG AAAATATACCTGTTTAAAAGC                       | KpnI        |
| cpsG_R01 | AACTGCAGTTTACCGGCAATTCGAGCTACCC                                           | PstI        |
| wcaJ_F02 | GGGTACCCCGgaggagAAACGATG ACAAATCTAAAAAGCGGAGGAG                               | KpnI        |
| wcaJ_R01 | AACTGCAGTTCA ATATCGGCTTGTAAACGAGACC                                      | PstI        |
| wzc_F01 | GGGTACCCCGgaggagAAACGATGACAGAAAAAGTAACACATGACG                                   | KpnI        |
| wzc_R01 | GCTCTAGATTTACGCGACCTTTATATGCGG                                       | XbaI        |
| wcaA_F01 | GGGTACCCCGgaggagAAACGATGAAAAACATCGGTACGTCACTCC                           | KpnI        |
| wcaA_R01 | GCTCTAGATTTACCGGCAATACACATCAG                                             | XbaI        |

On the sequence of the primers is represented: the restriction site (underlined); the Shine Dalgarno (lower case letter); start or stop codon (bold) for the forward (F symbol in the primer name) or reverse (R symbol) primers, respectively.

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(data not shown). Because of the depletion of histones H1 by repurification, we used the whole histones from the same batch for further experiments. We incubated the libraries with 25 µg/ml of histones for 3 h interrupted by a recovery of the culture by incubation in Luria Broth (LB) in presence of Micrococcal nuclease (MNase) for 2 h (Fig. 1A) and repeated the procedure 3 times. The MNase treatment was necessary to disperse bacteria that aggregated due to the released of DNA. We monitored the growth in the absence of selection. The bacterial suspensions measured the growth of the E. coli libraries in the same experimental conditions but in absence of histones, to determine the growth in the absence of selection. The bacterial suspensions incubated with histones showed fewer CFU/ml than the control cultures after the first recovery step although we washed and adjusted the suspensions to the same OD at each step. This difference confirms that histones were bactericidal (Fig. 1B). Notably, at the beginning of the third histone selection, the CFU/ml of the selected libraries was higher than before the second selection, indicating that fewer bacteria were dead at the beginning of the third selection. There were significantly more survivors in the pooled libraries than in E. coli carrying the empty vector when incubated with histones (Fig. 1B), confirming a positive selection of histone resistant bacteria.

After each selection we isolated clones from the pooled libraries incubated in the presence and in the absence of histones. The 10 clones isolated from the second selection were more resistant to histones than the 10 clones isolated from the libraries that were not under selection pressure (See Fig. S1). We sequenced 500–1000 bp of the 3’ and 3’ of their plasmid inserts, and, interestingly, a fragment including the wcaJ/I region (from genomic position 2

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**Table 1.** Primers designed for the different clonings of the wcal/J and wzc/wcaA fragments in pBAD33 and pBAD18 (see Fig. 2).
The proteins encoded by the colanic acid cluster are responsible for the production of the exopolysaccharide colanic acid ([22], Fig. 2A). In *E. coli* K-12 strain MG1655, this cluster can also contribute to the production of a short form of an O-antigen as well as Exopolysaccharide.

The resistance to histone of clones overexpressing genes involved in colanic acid synthesis could be explained by alteration in the barrier function of the outer membrane. With this in mind, we tested the susceptibility of different clones to detergents (sodium deoxycholate and SDS) and antibiotics (vancomycin, bacitracin and novobiocin). None of the clones tested showed any significant resistance, we determined the minimal bactericidal histone concentration to kill 90 (MBC90) or 99% (MBC99) of the bacteria inoculum in 1h after inducing over-expression with arabinose.

Over-expressing *wcaI*, *cpsB* and *cpsG* genes, in *E. coli* MACH1 resulted in high resistance to histones (MBC99 and 90±100 µg/ml) (Fig. 3A-B). These results validated our screening. Over-expression of two genes *wcaI/cpsB* or *cpsB/cpsG*, or *cpsB* alone also led to a high histone resistance (Fig. 3C–D) showing that *cpsB* is mainly responsible this phenotype. Interestingly, *cpsB* in combination with *wcaI* or *cpsG* conferred even a higher resistance as showed by the MBC90 (Fig. 3C) indicating synergy between these genes.

*wzc/wcaA* was the second fragment related to colanic acid identified in the screen. The MBC90 and 99 of the over-expression of *wzc* and *wcaA* or either one of those genes alone did not differ from the empty vector control (Fig. 3E–F). Intriguingly, the survival to a 1 h exposure to 100 µg/ml of histone was better in bacteria over-expressing *wzc* and *wcaA* or either gene alone than in cells carrying an empty vector (Fig. 3G). This low resistance to histone correlated with the low frequency of selection of these genes in our screen (Fig. 1D).

## Contribution of the Colanic Acid Cluster Genes to Histone Resistance

To determine the contribution of the different genes to histone resistance, we determined the minimal bactericidal histone concentration to kill 90 (MBC90) or 99% (MBC99) of the bacterial inoculum in 1h after inducing over-expression with arabinose.

Over-expressing *wcaI*, *cpsB* and *cpsG* genes, in *E. coli* MACH1 resulted in high resistance to histones (MBC99 and 90±100 µg/ml) (Fig. 3A-B). These results validated our screening. Over-expression of two genes *wcaI/cpsB* or *cpsB/cpsG*, or *cpsB* alone also led to a high histone resistance (Fig. 3C–D) showing that *cpsB* is mainly responsible this phenotype. Interestingly, *cpsB* in combination with *wcaI* or *cpsG* conferred even a higher resistance as showed by the MBC90 (Fig. 3C) indicating synergy between these genes.

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## Clones Over-expressing Genes in the Colonic Acid Pathway have a Normal Outer Membrane Barrier Function and Produce O-antigen as well as Exopolysaccharide

The proteins encoded by the colanic acid cluster are responsible for the production of the exopolysaccharide colanic acid ([22], Fig. 2A). In *E. coli* K-12 strain MG1653, this cluster can also contribute to the production of a short form of an O-
antigen [21]. We studied the production of exopolysaccharide and LPS in clones over-expressing the selected genes. The exopolysaccharide was extracted and isolated by differential precipitation steps. We failed to detect fucose colorimetrically in the extracts, as a measure of colanic acid [23]. Instead, we quantified the exopolysaccharide through its carbohydrate content. MACH1 strains over-expressing \textit{wcaI/cpsB}, \textit{cpsB/cpsG}, or \textit{cpsB} had more exopolysaccharide than strain carrying the empty vector (Fig. 4A).

We also analyzed the LPS of proteinase K-treated whole cell lysates by separation on polyacrylamide gels. \textit{E. coli} MACH1 carrying empty vector(s) showed mainly a rough LPS, consisting on lipid A and the core. The over-expression of \textit{wcaI/cpsB/cpsG} or \textit{cpsB} led to the production of a LPS of higher molecular weight (visible by the typical ladder pattern in the higher part of the gel), called semi- and smooth LPS and corresponding to the presence of different length of the O-antigen attach to the lipid A – core domains (Fig. 4B). Thus, the over-expression of \textit{cpsB} is the key factor for the production of an exopolysaccharide and an O-antigen+ LPS, which could explain the histone resistance.

The \textit{O}-antigen of \textit{K. pneumoniae} and \textit{S. flexneri} Protects Against Histone Bactericidal Activity

We determined the contribution of exopolysaccharide and LPS to histone resistance and in the pathogenic Gram-negative bacteria, \textit{S. flexneri} and \textit{K. pneumoniae}. Both of these species produce O-antigen+ LPS but only \textit{K. pneumoniae} is a capsulated. We compared the MBC90 and MBC99 of wild type \textit{K. pneumoniae} Kp52145 strain with the acapsulate mutant Kp52K10 [16]. \textit{K. pneumoniae} with or without capsule were resistant to histones (Fig. 5A), but the MBC90 of the acapsulate mutant is significantly higher than that of the wild type strain. The capsule seemed to have a limited impact on histone resistance. \textit{S. flexneri} is naturally acapsulated but O-antigen+, showed a higher histone resistance than MACH1 \textit{E. coli} carrying empty vectors (Fig. 5B, Fig. 3A–F). To address histone protection via
the O-antigen, we compared the wild type *S. flexneri* strain M90T with a *waaL* and a *rfbA* isogenic mutants (O-antigen-, [24] and manuscript in preparation: Geldmacher et al.)), as well as the wild type *K. pneumoniae* with the O-antigen mutant 52O21 [16]. The O-antigen- mutants in *S. flexneri* and *K. pneumoniae* backgrounds showed a significant higher sensitivity toward histones than the wild type strains (Fig. 5A–D). This last observation strongly suggests that the O-antigen is the main cell surface component to protect Gram-negative bacteria against histones.

**Discussion**

**Rationale of Using Over-expression Libraries**

Understanding how bacteria become more resistant or susceptible to AMPs can reveal their mode of action. One way to find out the mechanism of action of an AMP is to select susceptible clones to AMP(s) out of a mutant library. This approach has lead to a few interesting observations. For example, DhIA, a protein that alanylates lipoteichoic acid in the cell wall of the opportunistic Gram-positive pathogen *Staphylococcus aureus*, confers resistance to cationic AMPs [25]. Another example suggested that the neutralization of phosphatidylglycerol by lysine, mediated by *mprF*, also leads to AMP resistance [26]. Using a different AMP, Li et al. proposed that lipid modifications, dependent on *dak2*, also change the bacterial susceptibility [27]. It is likely that AMP target essential bacterial components, negating the use of this type of selection. Furthermore, like in any screen, if several genes have the same function, deletion only one of them has little, if any, impact on the phenotype. Taking essential genes and gene redundancy into consideration, we chose a different approach. We selected clones from homologous libraries of *E. coli* harboring low copy plasmids carrying genomic DNA fragment, where the genes are expressed under their native promoters. By having the genes under their native promoter but in several copies, the gene(s) would be over-expressed only slightly because of the low copy number plasmid and, since they are under their original promoter, physiologically regulated.

![Figure 2. Involvement of the selected genes in the colanic acid pathway and cloning under arabinose promoter.](image-url)

**(A)** The genes screened for histone resistance (bold pink) are represented in the scheme of the colanic acid pathway. The first steps of the colanic acid pathway consist on the production of activated sugar in the cytoplasm of the bacterium. Mannose-6-phosphate (Man-6-P) leads to the production to GDP-fucose by CpsG and CpsB. Others activated sugars, UDP-galactose (UDP-Gal), -glucosamine (-Glc) and –glucuronic acid (-GlcA), are produced also for other cellular components. The assembly of the first sugar unit, colanic acid (CA) unit, is assembled on the lipid carrier, called undecaprenyl pyrophosphate, by Wca proteins, such as WcaJ, WcaI and WcaA. Then the CA unit is exported and polymerized by the Wzc machinery, for the production of the exopolysaccharide. Another possibility is the addition of CA unit on the short form of the lipopolysaccharide (LPS) by an unknown mechanism [21,22].

**(B)** The selected genes belonging to the colanic acid cluster were cloned under arabinose promoter on pBAD33 and pBAD18 vectors. Different constructs were obtained to address different gene combinations in the histone resistance and to refine the essential genes for the resistance. (C) The toxicity of the arabinose-induced genes was tested by following the bacterial population (CFU/ml). After 2 h of culture (indicated by an arrow and considered as time 0), L-arabinose was added at 0.4 (open triangle, full line), 0.04 (light gray triangle, dash line) and 0.004% (dark gray triangle, dot line) final concentration or not (black diamond, full line), and the culture was prolonged during 6 h.

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Figure 3. Impact of over-expression of colanic acid related genes identified in the screen on the histone resistance. We determined the minimal bactericidal concentration of histones to kill 90% (MBC90, left panels) or 99% (MBC99, right panels) of the initial bacterial inoculum after 1 h of incubation. (A–B) The simultaneous over-expression of \textit{wcaI}, \textit{cpsB}, \textit{cpsG} genes in MACH1 carrying the plasmids pBAD33\textit{cpsG} pBAD18\textit{wcaI/cpsB} or pBAD33\textit{wcaI} pBAD18\textit{cpsB/cpsG} led to a high histone resistance in comparison with the strain carrying the empty vectors. (C–D) To refine the genes
Advantages of Selecting Resistant Clones

The underlying hypothesis of a positive selection is that, in a susceptible bacterium, AMPs interact with their target(s) at a certain molar ratio. Over-expression of the target(s) makes the bacterium resistant to the AMP. A parameter to consider is the efficacy of the AMP action. Histones, at MBC90 and over, kill E. coli contributing the resistance, we tested the over-expression of wcaI/cpsB, cpsB/cpsG, or single genes, wcaI, cpsB, and cpsG, carried by pBAD18. (E–F) The other set of genes related to the colanic acid, wzc/wcaA, did not show any significant histone resistance in term of MBC90 and MBC99 in comparison with the bacteria carrying the empty vector, while the genes were over-expressed together (MACH1 pBAD18wzc/wcaA) or individually (MACH1 pBAD18wzc and MACH1 pBAD18wcaA). (G) The E. coli over-expressing this different combination of wzc and wcaA genes showed a higher number of survivors after 1 h treatment with histones at 100 μg/ml. To be able to compare the different clones, we standardized the values by dividing the CFU/ml after 1 h treatment by the CFU/ml of the inoculum. Each dot represents the result of one experiment and at least three independent experiments have been done. The differences were considered significant by the Mann-Whitney test with p≤0.05 (*) and p≤0.01 (**).

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Figure 4. Characterization of exopolysaccharide and lipopolysaccharide production in E. coli MACH1 expressing wcaI, cpsB and cpsG genes. (A) We purified the exopolysaccharide from supernatants of overnight liquid culture E. coli MACH1 pBAD18 empty vector or carrying wcaI/cpsB, cpsB/cpsG, wcaI, cpsB or cpsG, growing in presence of arabinose. The isolated exopolysaccharide was quantified by determining the carbohydrate content. Each dot represents the result of one measurement. The differences were considered significant by the Mann-Whitney test with p≤0.001 (***). (B) From the same liquid cultures, an aliquot was taken to analyze the LPS. The preparations of proteinase K-treated whole cell lysates were run on a Tris-tricine polyacrylamide gel and silver stained after periodate treatment. The gel represents an example of 3 independent experiments.

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coli efficiently in less than 10 min. (Data not shown, [5]). Even though the bactericidal action is fast, it could induce bacterial response at a transcriptional level. Thus, over-expression libraries contain clones which should be already resistant even in the absence of the AMP, and some which would become resistant by regulation on the native promoter of the gene(s). Experimentally, a selection of resistant clones allows also a reliable and fast read out because the interesting clones will survive or even grow in the presence of the AMP, as we could observe while selecting with histones. Our selection procedure was validated by several controls. One set of controls was to follow the bacterial survival of the library in the presence of absence of the selection pressure, as well as the incubation of bacteria carrying the empty vector in presence with histones.

Suitable Screening Approach for AMPs

The different histones could have different mode of actions. For example, histone H1 shows different biochemical properties than the core histones (pI, sequence, structure) suggesting a distinct bactericidal mechanism than the others histones. Nevertheless, we chose to perform our screen with mixture composed of 5 histones in vivo bacteria are likely to encounter a complex variety of proteins. Furthermore, the histones we used are post-translational modified increasing the diversity of the proteins.

Toxicity Due to the Over-expression of wcaJ

WcaJ is predicted to be the glycosyltransferase which loads the first sugar of what will become a polysaccharide on a lipid carrier, the undecaprenyl pyrophosphate ([22], Fig. 2A). This lipid carrier is a common transporter for precursors of essential bacterial cell wall components, such as LPS and peptidoglycan [28]. High amount of WcaJ in the bacterium might unbalance the use of the lipid carrier and the production of polysaccharide synthesis. The decrease of peptidoglycan synthesis could inhibit bacterial growth or be lethal to the bacterium, as we observed when over-expressing wcaJ. The co-expression of other downstream enzymes in the

![Figure 5. Contribution of capsule and LPS in histone resistance in Klebsiella pneumoniae and Shigella flexneri.](https://doi.org/10.1371/journal.pone.0071097.g005)

MBC90 and MBC99 have been determined for (A–B) K. pneumoniae wild type (Kp52), acapsulated mutant (52K10) and O-antigen mutant (52O21), and (C–D) in S. flexneri background: wild type (M90T) and O-antigen mutant (waaL-). Each dot represents the result of one experiment and at least 4 independent experiments have been done. The differences were considered significant by the Mann-Whitney test with p$<0.05$ (*).
synthesis of the polysaccharide, such as WcaI, would increase the production of polysaccharide and induce bacterial lethality. This could explain our observation on the bactericidal effect of concomitant over-expression of the wcaI/cpsB/cpsG/wcaJ genes. Moreover, it is known that growth rate, and in particular the presence of a strong proton motive force, can have an influence on the relative toxicity of different antibiotics, including AMPs [29]. In our studies, this may have played a role in the observed protection of slow growers, even though, the histone bactericidal activity is not affected by bacteriostatic conditions (unpublished data). However, in the case of over-expressing cpsB alone or in combination with cpsG or wcaI, we did not observe decreased in growth rate, and did confirm the production of an O-antigen thus suggesting that reduced growth rate did not play a major role in our observed protection.

Capsule and Histone Killing

We selected genes involved in the production of the colanic acid in E. coli MG1655. In the over-expressing E. coli MACH1 clones, we quantified a significant production of an exopolysaccharide based on a carbohydrate determination. But in the different exopolysaccharide extracts we could not detect fucose using the sulfuric acid reaction, which is one of a typical sugar for the colanic acid exopolysaccharide. We can speculate that the over-expression of cpsB restored the LPS and exopolysaccharide pathways in E. coli MACH1.

Since we selected genes required for the production of an exopolysaccharide, we hypothesized that a bacterial capsule should also play a barrier role for histones. The capsule of K. pneumoniae prevents killing by human neutrophil alpha-defensin 1, polymyxin B or protamine, but not to complement activity [30]. Surprisingly, in our assays, the capsule of K. pneumoniae is not protective towards histones. The capsule may just delay the interaction with the bacterial cell wall but does not abrogate it. We could speculate that the absence of the capsule, which is not truly a barrier for histones, unmasked the O-antigen possessing better properties to interfere with the interaction of histones with the bacterial membrane.

Interaction of Histones with Bacterial LPS

The first step of cationic AMPs, probably including histones, is to interact with the bacterial surface via electrostatic interaction [2,31,32]. Modifications of surface components for Gram-negative and -positive bacteria have been described to protect pathogens against a variety of AMPs by abrogating their interaction with the bacterial surface [2,33]. One example of LPS modification is the addition of 4-amino-4-deoxy-l-arabinose on the lipid A, target of polymyxin [34]. This is consistent with our results, where we selected for genes related to O-antigen and exopolysaccharide synthesis. The O-antigen neutralized the negative charge of the bacterial surface decreasing the attractive properties toward cationic AMPs [2,33]. In the case of S. flexneri, we showed that the presence of the O-antigen lead to the neutralization of the bacterial surface (manuscript in preparation: Geldmacher et al.). Moreover, it has been shown that histones interact with the lipid A in vitro, portion embedded in the outer membrane of Gram-negative bacteria [35]. Thus, we could speculate that the O-antigen could mask the lipid A and avoid the binding of histones on it.

To conclude, the electrostatic interactions of histones to the bacterial cell wall would be the first step of their mode of action, which will be common to Gram-negative and -positive bacteria. We could also speculate that the binding of histone to the lipid A of the LPS would lead to death of Gram-negative bacteria. It has been shown that LPS protect bacteria towards different AMPs [2,33]. Moreover, these bacteria may have been confronted by a variety of histones present in intact or partial forms during their evolution or in a more recent history. Thus it is reasonable that histone protection may have played a role in the selection of current LPS properties.

Supporting Information

Figure S1 Growth curves of E. coli clones pre-incubated with 100, 25, 10 and 0 μg/ml final of calf thymus histones (annotated H100, H25, H10 and H0 and represented by a pink, red, orange and blue curves, respectively). Single clones isolated during histone selection were tested for their histone resistance or susceptibility. As controls, we tested 10 E. coli clones isolated from the non-selected libraries and E. coli MACH1 pSMART-LCKm (empty vector). The bacterial suspensions were adjusted to final concentration of 10^8 bacteria/ml in HAH medium (HBSS-, casa-amino-acid 0.9% and HEPES 10 mM) supplemented with Km 50 μg/ml. The clones were incubated with 100, 25, 10 and 0 μg/ml final of calf thymus histones 1 h at 37°C with agitation. Then, the bacterial growth was allowed by adding 1 volume of 2X LB, CaCl2 10 mM and Km 50 (37°C with agitation). The growth was followed in 96 well-plate by reading the absorbance at 600 nm during 10 h with a reading every 5 min. The growth curves would be dependent of the amount of living bacteria at the end of the incubation with histone, as shown by the profiles of the MACH1 pSMART-LCKm and the isolated clones from the non-selected libraries. The 10 clones isolated from the selected libraries showed higher resistance. SL2+ corresponds to the 1 to 10 clones isolated from the histone-selected libraries. SL2- corresponds to the 1 to 10 clones isolated from the non-selected libraries. MACH1 pSMART-LCKm is the control of E. coli harboring the empty vector. (PDF)

Table S1 Data from the microarray analyses. The genes are ranked in function of their fitness as described in the manuscript. (XLSX)

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Author Contributions

Conceived and designed the experiments: CC RTG AZ. Performed the experiments: CC ES. Analyzed the data: CC. Contributed reagents/materials/analysis tools: CC ES RTG. Wrote the paper: CC.

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