Antimicrobial Components of the Neonatal Gut Affected Upon Colonization

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ABSTRACT: Antimicrobial peptides (AMP) produced throughout our body are important effectors in the defense barrier of innate immunity. Here, we have analyzed antimicrobial activity and polypeptide composition of meconium *versus* neonatal feces to address the development of antimicrobial defense of the neonatal gut. Extracts of meconium exhibited antimicrobial activity against *Bacillus megaterium*, *Escherichia coli*, and group B streptococci (GBS) but not against the yeast *Candida albicans*. Extracts of neonatal feces were found to possess low activity against *E. coli*, GBS, and *C. albicans*. However, the anti-*B. megaterium* activity was higher in the fecal extracts than in meconium. All activities were reduced or abolished when salt was added to the antimicrobial assay. The AMP cathelicidin LL-37, α-defensin HNP-1-2, α-defensin HD 5, and lysozyme were identified in both meconium and fecal extracts. In addition, HNP-3 and a fragment of azurocidin were found in meconium, whereas the holoprotein azurocidin was detected in feces. In meconium, histones H2A and H4 were isolated and identified by their antimicrobial activity. Notably, LL-37 and lysozyme were found at significantly higher levels in feces than in meconium. Our findings reveal that meconium and feces contain AMP, acting in the defense of the neonatal gut, and may be implicated in the control of the initial colonization. (*Pediatr Res* **61:** 530–536, 2007)

Meconium is a sterile mucilaginous material that accumulates in the fetal intestine and is expelled soon after birth. It contains secretions of intestinal glands, gut constituents (proteins, bile acids, fatty acids, and steroids), and components of amniotic fluid and vernix caseosa (1). In addition, meconium has been reported to contain proinflammatory substances, including IL-1β, IL-6, and IL-8, compounds that are able to induce immune responses such as activation of lymphocytes and chemotaxis of neutrophils (2,3). The first microbes to colonize the intestines of newborn babies are the aerobic bacteria *Escherichia coli* and *Streptococci*. Later, the gut is colonized with anaerobic bacteria, *e.g. Bacteroides*, *Bifidobacteria*, and *Clostridia*. In 1–2 y, the gastrointestinal tract of infants has developed a natural microflora, which resembles the microflora of adults (4).

Despite the fact that newborns have a naive adaptive immune system, infections rarely occur, indicating strong innate defense mechanisms. Several AMP have been characterized in vernix and skin of the newborn, indicating a well-developed innate immune system, which may play a pivotal role at the time of postnatal colonization. AMP are important effectors of innate immunity with the ability to kill a wide range of microbes (5). The defensins and cathelicidins are the two main families of human AMP (6,7). They are expressed at epithelial linings, *i.e.* the adult human gastrointestinal tract, where they are either constitutively expressed or up-regulated by bacterial factors/products such as lipopolysaccharides and butyrate (8–11).

In the alimentary tract, polypeptides with bactericidal activities are also present, such as lysozyme, phospholipase A2, different histones, and ribosomal proteins, which have been isolated from adult colonic epithelium and mucosa (12,13). This complex mixture of AMP in colon indicates that the gut is protected against potential human pathogens. A regulatory function of the natural flora has also been suggested for AMP, which may be of significant importance in the neonatal gut (14).

To address whether AMP are produced already in the fetal gut where no microbes are present, meconium was analyzed and found to exhibit activity against both Gram-negative and -positive bacteria. Hence, we show that meconium contains several AMP, indicating the capability of the fetal gastrointestinal tract to produce such factors. However, because bacterial components are known to be important activators of several AMP genes, we hypothesized that this defense system is induced upon colonization. Therefore, we analyzed the antimicrobial activity and polypeptide composition of feces sampled 3–8 d after birth. Our results reveal that the feces samples, compared with meconium samples, exhibited higher

Abbreviations: AMP, antimicrobial peptides; **GBS,** group B streptococci; **HD,** human defensin; **HNP,** human neutrophil peptide; **MALDI-MS,** matrix-assisted laser desorption/ionization mass spectrometry; **RP,** reversed phase; **TFA,** trifluoroacetic acid; **WCEX,** weak cationic exchange chromatography

Table 1. *Neonates included in the study and the presence of LL-37, azurocidin, and an azurocidin fragment in the respective samples*

Neonate no.	Gestation age (wk)	Gender	Birth weight	Postnatal age (h or d)	Polypeptide present (+) or absent (−)		
					LL-37	Azurocidin	Azurocidin fragment (4 kDa)
Meconium							
1	36	Male	2880	24h	ND	ND	ND
2	39	Female	3530	8h	−	ND	ND
3	38	Male	3150	13h	−	−	+
4	35	Male	3200	24h	ND	ND	ND
5	39	Male	4270	18h	+	−	+
6	37	Male	2790	47h	+	−	+
7	41	Male	3900	18h	+	−	+
8	39	Female	3490	12h	+	−	+
9	38	Female	3140	15h	−	−	+
10	37	Female	3010	20h	+	−	+
13‡	41	Male	3550	22h	+	−	+
14‡	39	Male	3300	8h	+	+	+
15‡	41	Female	3600	7h	+	−	+
16‖	40	Female	3620	18h	−	ND	ND
17‖	40	Male	3520	6h	+	ND	ND
18‖	41	Female	2915	6h	−	ND	ND
19‖	38	Female	3390	27h	−	ND	ND
20‖	40	Male	3480	19h	−	ND	ND
Feces							
11	40	Female	3580	4d	+	ND	ND
12	40	Male	4100	5d	+	+	+
13‡	41	Male	3550	4d	+	+	−
14‡	39	Male	3300	3d	+	−	−
15‡	41	Female	3600	4d	+	+	−
16‖	40	Female	3620	8d	+	ND	ND
17‖	40	Male	3520	5d	+	ND	ND
18‖	41	Female	2915	4d	+	ND	ND
19‖	38	Female	3390	8d	+	ND	ND
20‖	40	Male	3480	8d	+	ND	ND

ND, not determined.

Extract (100 or 650 μg) was analyzed except for those originating from neonates marked with ‡ or ‖, where 5 μg or 15 μg protein was analyzed, respectively.

antimicrobial activity and contained significantly higher levels of some AMP.

METHODS

Samples. Meconium and feces samples were collected from diapers of 20 healthy neonates, born at term gestation after uncomplicated pregnancy and delivery (Table 1) and exclusively breast fed. None of the mothers received antibiotics before, during, or after delivery. The parents gave informed consent for their infant to participate in the study. The collected samples were immediately frozen at –20°C. Proteins were extracted and enriched from the meconium and feces samples as previously described for vernix samples (15) and quantified with the Bradford method (Bio-Rad, Hercules, CA).

Inhibition zone assay. The antimicrobial assay and the detection of lysozyme were performed according to a modified protocol as previously described (15). As test microbes, *Bacillus megaterium* (strain BM11), *Escherichia coli* (strain D21), *Candida albicans* (strain ATCC 1443), and a clinical isolate of group B streptococci (GBS), verified by a Phadebact Strep B test (Boule Diagnostic AB, Stockholm, Sweden), were used. All assays were performed with and without medium E (16).

Pepsin digestion. Incubation of 150 μg extracts was carried out for 5 h at 37°C in 5% formic acid with 75 μg pepsin (Sigma Chemical Co., St. Louis, MO). After lyophilization, the digests were dissolved in 3.5 μL 0.1% TFA and analyzed with the inhibition zone assay without medium E.

Isolation of AMP. WCEX was performed as previously described (17). Two different gradients of 1 M or 1.5 M ammonium acetate in 0.2 M acetic acid were used, *i.e.* 0–50% in 30 min, followed by 50–100% in 50 min, and 0–20% in 10 min, followed by 20–100% in 80 min. Fractions containing antimicrobial activity were further loaded on an Ace C$_{18}$ column (5 μm; 150 × 2.1 mm; Advanced Chromatography Technologies, Aberdeen, Scotland). The C$_{18}$ column was equilibrated in 0.1% TFA and elution was carried out with a linear gradient of acetonitrile containing 0.1% TFA at a flow rate of 0.2 mL/min.

SDS-Page. Extracts/fractions were analyzed with SDS-PAGE utilizing 10–20% Tricine gels or NuPAGE gels (Invitrogen, Carlsbad, CA), which were stained with SilverXpress (Invitrogen) or used for Western blot analysis.

Western blot and dot blot analyses. The presence of LL-37, azurocidin, and HNP-1-3 in extracts/fractions was determined with Western blot or dot blot analyses as previously described (15). The following concentrations of the primary antibodies were as follows: MAb specific for LL-37, 0.6 μg/mL; polyclonal antibodies directed to azurocidin, 2 μg/mL; and MAb for HNP-1-3, 0.2 μg/mL. The concentrations of the secondary antibodies (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) were anti-mouse IgG (1:5000) for the MAb and anti-rabbit IgG (1:5000) for the polyclonal antibodies. Immunoreactive protein bands were visualized with ECL-plus (Amersham Pharmacia Biotech, Inc.).

Trypsin digestion and peptide mass fingerprinting. Aliquots of HPLC fractions were lyophilized, redissolved in 0.25 M ammonium bicarbonate (pH 8), and treated with 10 μg/mL trypsin for 4 h at 37°C. Fragments were analyzed by MALDI-MS and proteins were identified by database searches using the ProteinProspector MS-Fit program (http://prospector.ucsf.edu/) on the mass values of the tryptic fragments (18).

Protein identification. Material in HPLC fractions and tryptic fragments thereof were analyzed by MALDI-MS and/or amino acid sequence analysis as described previously (19).

Statistical methods. Statistical differences between groups were assessed with two-tailed nonparametric test Mann-Whitney test with a 95% confidence interval. *p* Values below 0.05 were considered significant.

RESULTS

Antimicrobial activity in meconium and feces extracts. The lyophilized meconium extracts were dissolved in 0.1% TFA and contained 0.5–7.2% (wt/wt) proteins. These extracts

A

B
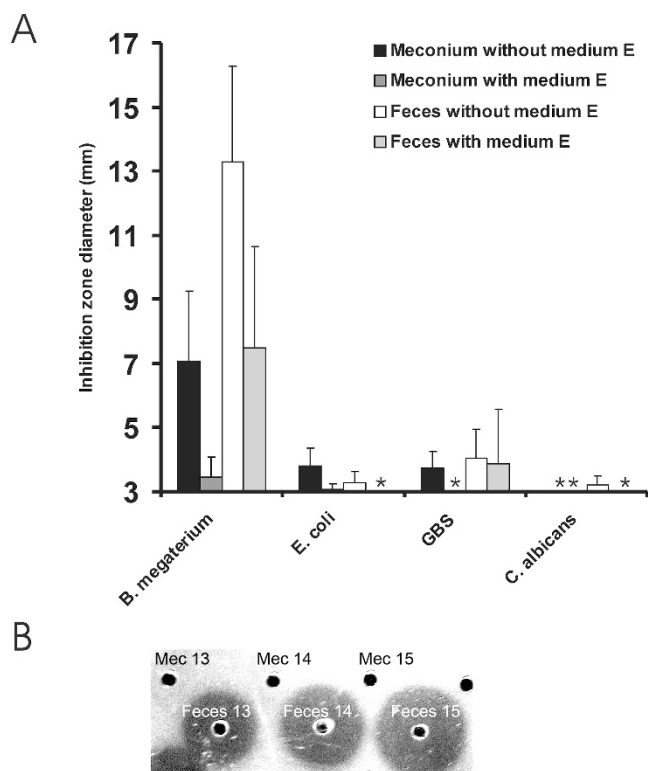
Figure 1. Antimicrobial activities in extracts of meconium and feces. (*A*) The activities against *B. megaterium*, *E. coli*, GBS, and *C. albicans* were analyzed with and without medium E. Each column representing meconium is the average of 14 samples. Due to limitations in the amount of material, nine samples were measured in triplicates, two samples in duplicates, and three samples as single measurement. Each bar representing fecal activity is the average of nine samples. Three of these samples were measured in triplicates, and six of the samples in duplicates. The error bars indicate the SEM. *No activity was recorded against the microbe of interest under the given conditions. (*B*) The lysozyme activity is shown in meconium and fecal samples originating from three neonates (neonates 13–15). The zones were recorded after one night incubation and the assay was repeated twice.

(150 μg) were assayed for antimicrobial activity against *B. megaterium*, *E. coli*, GBS, and *C. albicans* and exhibited antimicrobial activity against all microbes tested except *C. albicans* (Fig. 1*A*). When medium E was included in the assay, the activity against *B. megaterium* was reduced ($p < 0.0001$), whereas the activities against *E. coli* and GBS were abolished (Fig. 1*A*). Pepsin degradation of the meconium extracts gave a slightly decreased activity against *B. megaterium* (9%). However, it did not alter the activity against GBS and it caused an increase of the anti-*E. coli* (18%) activity (data not shown).

Fecal extracts (150 μg), containing 4.8–24% (wt/wt) proteins, exhibited significantly higher activity against *B. megaterium* than meconium both with and without medium E ($p = 0.0001$ and $p = 0.0002$, respectively). However, the activities against *E. coli*, GBS, and *C. albicans* were low (Fig. 1*A*). As for meconium, medium E reduced most of the activity against *B. megaterium* ($p = 0.0028$), and depleted the low activity against *E. coli* and *C. albicans*. After pepsin digestion, the activity against *B. megaterium* was dramatically reduced (58%) (data not shown).

To detect and compare the lysozyme activity, eight meconium and fecal extracts, representing 5 μg protein, originating from the same newborn, were analyzed in two assays. After one night of incubation, the fecal samples exhibited significantly higher activity than the corresponding meconium samples ($p = 0.0002$), with zones ranging from 8.2 to 18.2 mm in diameters, whereas the zones of the meconium samples ranged from 3.0 to 4.6 mm (Fig. 1*B*).
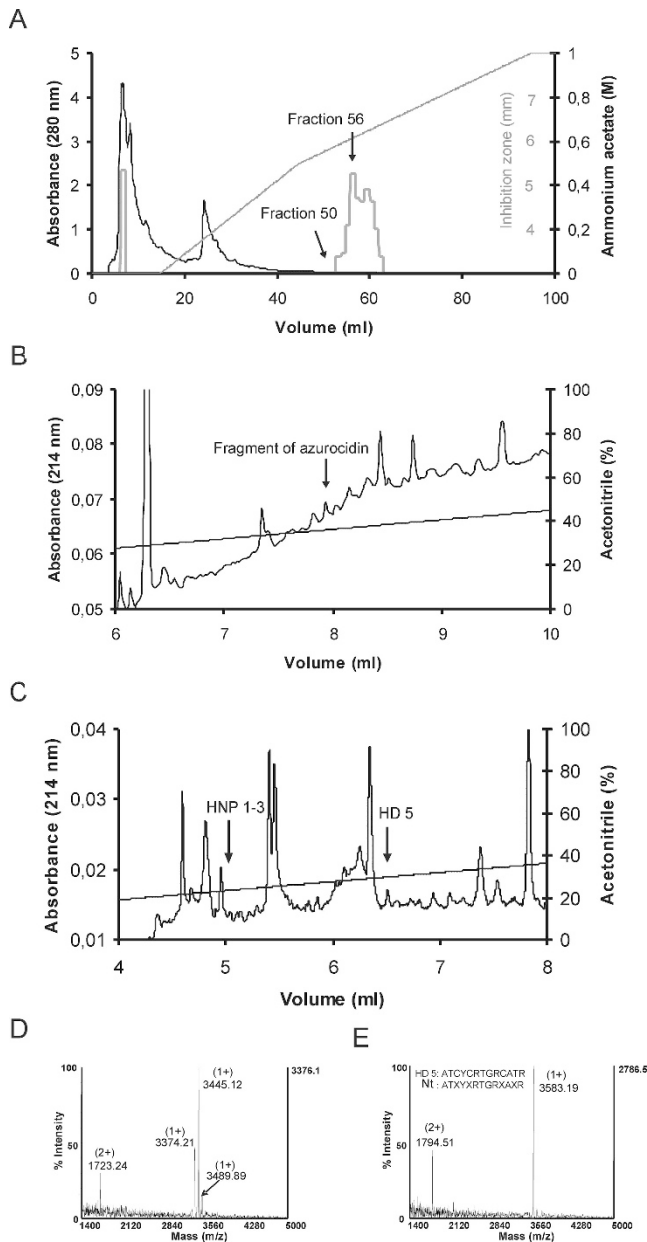
Isolation and identification of AMP in meconium. Meconium extract (20 mg) was loaded onto a WCEX column. Fraction 7 and fractions 53–62 exhibited activity against *B. megaterium* when analyzed with the inhibition zone assay, where medium E was included. Fraction 56, exhibiting the highest activity, eluted at 0.6 M ammonium acetate (Fig. 2*A*). Material in this fraction was further purified by reversed phase (RP) chromatography (Fig. 2*C*) and screened for mass values that correspond to known AMP. The fraction eluting at 23% acetonitrile contained the α-defensins human neutrophil peptides (HNP) of types 1–3 as identified with MALDI-MS (Fig. 2*D* and Table 2). The HD type 5 was identified in the fraction eluting at 29% acetonitrile with MALDI-MS and N-terminal sequence analysis for 13 cycles (ATXYXRTGRXAXR) (Fig. 2*E* and Table 2).

The fraction eluting at 0.54 M ammonium acetate (fraction 50 in Fig. 2*A*) from the WCEX-HPLC was further separated using RP chromatography (Fig. 2*B*). With N-terminal sequence analysis of the material eluting at 37% acetonitrile, the sequence GRXAXPRQFPFLASI was obtained. This sequence shared significant identity with residues 4–18 of the antimicrobial polypeptide azurocidin. Further analysis with MALDI-MS gave molecular weights of 4048 Da and 4054 Da. The theoretical mass value of 4041 Da, corresponding to residues 4–39 of the mature azurocidin protein, is the closest to the observed mass values.

To scale up the purification procedure, 45 mg meconium extract was separated on WCEX-HPLC. The fractions were screened for anti-*B. megaterium* activity without addition of medium E in the assay and activity was detected in fractions 5–9, 27, and 53–81. Material eluting at 0.80–0.84 M ammonium acetate (fractions 60–62 in Fig. 3*A*), was pooled and submitted to RP-HPLC (Fig. 3*B*). When material in fractions eluting at 47% and 51% acetonitrile, was loaded onto a tricine gel, a single polypeptide band was visualized in each fraction (inset in Fig. 3*B*). More accurate molecular weights, 11.3 kDa and 13.9 kDa, were obtained with MALDI-MS (Table 2). Attempts to determine the N-terminal sequence of these polypeptides failed, suggesting that they were N-terminally blocked. Hence, they were identified with peptide mass fingerprinting, and found to be histone H4 and H2A, which are indeed N-terminally blocked (20). Histone H4 was identified with eight matching fragments in 62% total sequence coverage, and histone H2A with seven matching fragments in 40% total sequence coverage.

Isolation and identification of fecal AMP. An isolation protocol, identical to that of 20 mg meconium extract, was used for 20 mg feces extract to detect the same polypeptides in the respective material. Anti-*B. megaterium* activity was detected in fractions 21–25 and 36–96 from the WCEX-

eluting at 34% acetonitrile (Fig. 4*B*) and identified with both MALDI-MS (Fig. 4*D*) and dot blot analysis.

Immunodetection of LL-37 in meconium and feces. To address whether the LL-37 level is altered after colonization, 5 μg (neonates 13–15) or 15 μg (neonates 16–20) protein samples, originating from both meconium and feces of the same neonates, were analyzed. In six of eight fecal samples, LL-37 and/or its proform were present at higher levels than in the corresponding meconium sample (Fig. 5*A*). Furthermore, additional eight meconium samples and two feces samples from noncorresponding newborns were analyzed for the presence of LL-37 with Western blot analysis (100 μg extract). A faint immunoreactive band of 4.5 kD corresponding to LL-37 was detected in five meconium extracts. Furthermore, LL-37 and its proform (17 kD) were detected in both feces extracts (Table 1).

Immunodetection of azurocidin and an azurocidin fragment in extracts of meconium and feces. As for LL-37, the azurocidin levels in meconium and feces were compared with Western blot analysis. Three extracts (5 μg protein) of both meconium and feces derived from the same neonates were analyzed. Two of the fecal samples and one meconium samples contained azurocidin (Table 1). Moreover, the presence of azurocidin was analyzed in 7 meconium samples (650 μg extract) and 1 fecal sample (100 μg extracts) from different newborns. In the feces sample, azurocidin (37 kD) was detected. In all meconium extracts a clear immunoreactive band with a mobility corresponding to a molecular weight close to 4 kD was observed, confirming the presence of a fragment of azurocidin (Fig. 5*B*, Table 1) as isolated and characterized (Fig. 2*B*).

DISCUSSION

In this study, we show that meconial and fecal extracts exhibit antimicrobial activity. We have also identified AMP in these extracts, reflecting their presence in the fetal/neonatal gut. In addition, some of these peptides are further induced, most probably in response to postnatal colonization of the gastrointestinal tract.

Meconium extracts were found to be active against *B. megaterium*, *E. coli*, and GBS. Notably, *E. coli* and GBS are among the first microbes to colonize the gut where they become a part of the commensal flora. However, if our host defense mechanisms are not able to control the growth of these bacteria, they can cause disease. The newborns are immunologically naïve, therefore GBS and *E. coli* are common pathogens of infants (4,21). The microbicidal properties of meconium suggests that it has a protective role in the neonatal gut and may define the set point of microbial colonization. The antimicrobial defense mechanisms might be crucial for the fetus in its transition from the sterile environment of the uterus to a world rich in bacteria. Fecal extracts were found to be significantly more active against *B. megaterium* than meconium and possess activity against all microbes tested. This indicates that the production of antimicrobial substances is triggered upon contact with microbes present in the gut after birth.


Figure 2. Isolation and identification of AMP from 20 mg meconium extract. (*A*) The initial step was performed utilizing WCEX-HPLC and the fractions were lyophilized and dissolved in 30 μL 0.1% TFA before analyzing the antimicrobial activity. The anti-*B. megaterium* activity of the material in the fractions is shown by the gray line. (*B*) The material in fraction 50 was further loaded onto a RP column and a fragment of azurocidin was identified with N-terminal sequence analysis and MALDI-MS in the fraction eluting at 37% acetonitrile. (*C* and *D*) The most active material (fraction 56 in *A*) was loaded onto a RP column and at 23% acetonitrile HNP-1 (3444 Da), HNP-2 (3373 Da), and HNP-3 (3489 Da) were identified with MALDI-MS. (*E*) In fraction eluting at 29% acetonitrile (in *C*), HD 5 was identified with N-terminal sequence analysis (Nt) and the mass value was determined with MALDI-MS (3582 Da).

HPLC when analyzed without medium E (Fig. 4*A*). The active fractions 80–84 eluting at 0.84–0.89 M ammonium acetate (Fig. 4*A*) were pooled. This material was further separated, utilizing RP-HPLC (Fig. 4*B*). In the fraction eluting at 29% acetonitrile HD 5 (Fig. 4*B*) was identified with MALDI-MS (Fig. 4*C*). In addition, HNP-1-2 were isolated in a fraction

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| Component (accession no.)	WCEX-HPLC elution (M ammonium acetate)	RP HPLC elution (% acetonitrile)	Method of identification	Theoretical mass/ determined mass
Meconium				
HNP-1 (P59665)	0.60	23	MS	3442 Da/3444 Da
HNP-2 (P59665)	0.60	23	MS	3371 Da/3373 Da
HNP-3 (P59666)	0.60	23	MS	3486 Da/3489 Da
HD 5 (Q01523)	0.60	29	MS, NTSA	3582 Da/3582 Da
LL-37 (P49913)	NA	NA	WBA	4493 Da/4.5 kDa
Histone H2A (Q99878)	0.80–0.84	51	PMF	13.9 kDa/13.9 kDa
Histone H4 (P62805)	0.80–0.84	47	PMF	11.4 kDa/11.3 kDa
Lysozyme (P61626)	NA	NA	*M. lysodeikticus**	16.5 kDa/NA
Azurocidin fragment (CAA41601)	0.54	37	MS, NTSA, WBA	4041 Da/4048, 4054 Da
Feces				
HNP-1 (P59665)	0.84–0.89	34	MS, dot blot analysis	3442 Da/3448 Da
HNP-2 (P59665)	0.84–0.89	34	MS, dot blot analysis	3371 Da/3376 Da
HD 5 (Q01523)	0.84–0.89	29	MS	3582 Da/3584 Da
LL-37 (P49913)	NA	NA	WBA	4493 Da/4.5 kDa
Lysozyme (P61626)	NA	NA	*M. lysodeikticus*	16.5 kDa/NA
Azurocidin (CAA41601)	NA	NA	WBA	37 kDa/37 kDa

MS, mass spectrometry; NTSA, N-terminal sequence analysis; WBA, Western blot analysis; PMF, peptide mass fingerprinting; NA, not applicable.
*See method for lysozyme detection.

The meconial and fecal bactericidal activities were reduced or abolished when salt was added to the inhibition zone assay. This suggests that the majority of the antimicrobial components in both meconium and feces are salt-sensitive. Examples of AMP possessing lower antimicrobial activity at high salt concentrations are the α-defensins HNP and HD 5 (22,23),
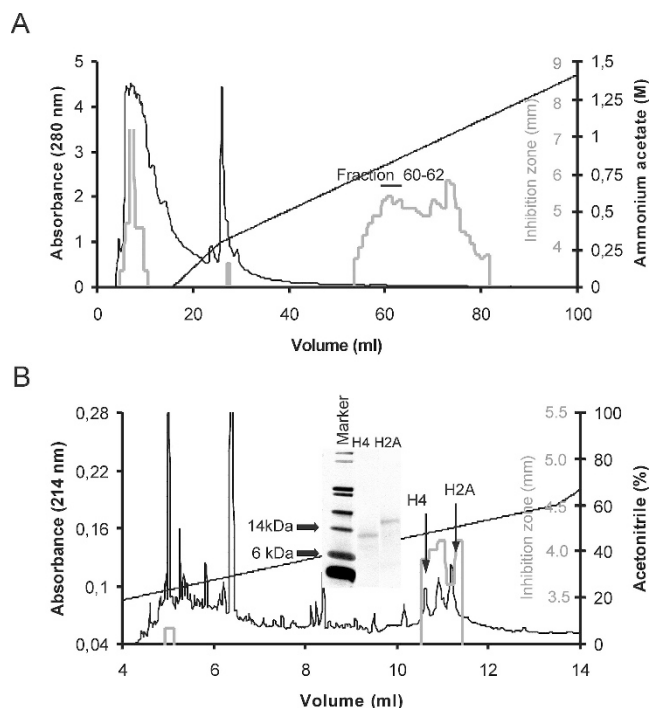
Figure 3. Purification and characterization of AMP from 45 mg meconium extract. (*A*) The initial fractionation was performed using WCEX-HPLC. After lyophilization, each fraction was dissolved in 65 μL 0.1% TFA. The material in the fractions was screened against *B. megaterium* and the activity is indicated by the gray line. Fractions 60–62 containing active material was loaded onto a RP column. (*B*) The active fractions 44 and 48 contained single protein bands, detected with SDS-PAGE (*inset*). Those proteins were identified as histone H4 and H2A by utilizing peptide mass fingerprinting and MALDI-MS.
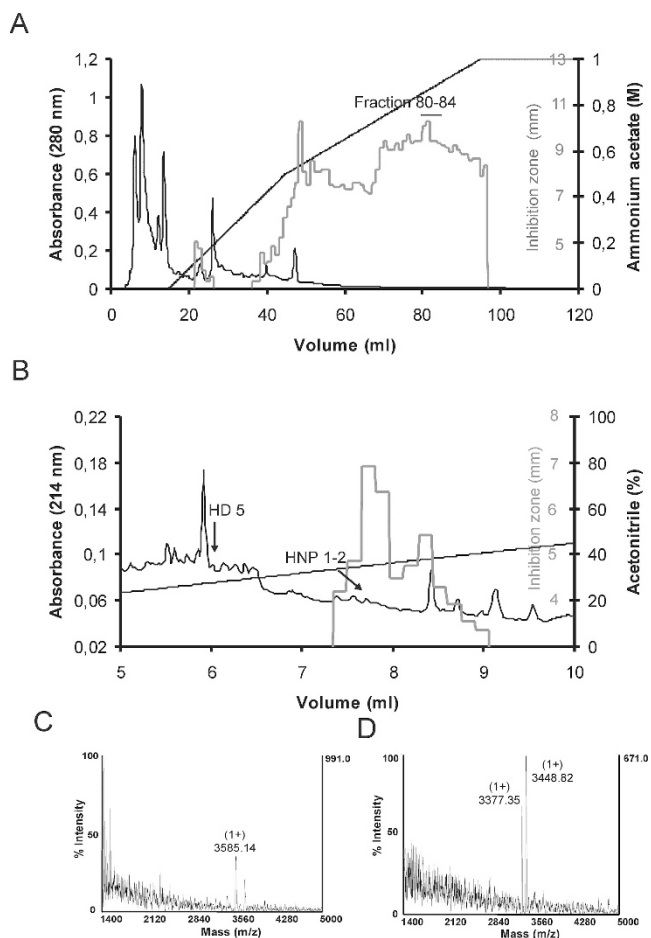
Figure 4. Isolation and identification of AMP in feces extract. (*A*) The first separation was performed with WCEX-HPLC and after lyophilization each fraction was dissolved in 30 μL 0.1% TFA. The anti-*B. megaterium* activity was monitored and is indicated by the gray line. (*B*) The active fraction 80–84 were pooled and loaded onto a RP column. HD 5 eluted at the same percent acetonitrile (29%) in *B* as in the chromatography of meconium, and (*C*) was identified with MALDI-MS. HNP-1-2 eluted at 34% acetonitrile in *B* and (*D*) were identified with MALDI-MS together with dot blot analysis.
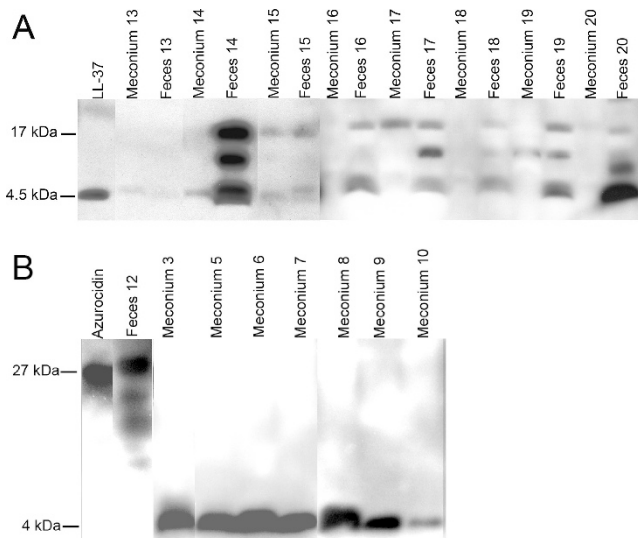
Figure 5. Detection of LL-37 and azurocidin in meconium and neonatal feces extracts. (*A*) Protein material (5 or 15 μg) was derived from extract of eight neonates and was analyzed with Western blot analysis. Immunoreactive bands corresponding to LL-37 (4.5 kD) and its proform (17 kD) were detected in both meconium and feces samples and their levels varied between the individual neonates. In six of the eight fecal samples (neonates 14 and 16–20), LL-37 was present at higher levels than in the corresponding meconium sample. (*B*) Extracts of seven meconium samples and one fecal extract were analyzed with Western blot analysis. An immunoreactive band corresponding to azurocidin (37 kD) together with additional fragments of different sizes were found in the fecal sample using the azurocidin antibodies. In all meconium samples, an immunoreactive band corresponding to a 4-kD fragment of azurocidin was detected. As positive controls, (*A*) 0.5 ng of synthetic LL-37 and (*B*) 40 ng of recombinant human azurocidin were used. The difference in molecular weight between the recombinant and native azurocidin can be explained by more glycosylation in the native protein.

here identified in both meconium and feces. However, also salt-induced AMPs, such as LL-37, have here been identified in the material analyzed. Salt promotes the alpha-helical conformation of LL-37, which is necessary for its antimicrobial properties (24). Thus, reduced activity of meconium and feces extracts in the presence of salt indicates that the major contributor to the microbicidal activity may be the defensin-like peptides. However, the existence of LL-37 supports that the defense mechanism is active in different conditions.

After pepsin digestion of the fecal extracts, the anti-*B. megaterium* activity was dramatically decreased. In contrast, meconium extracts treated with pepsin did not show any pronounced reduction of the antibacterial activity. This indicates that components other than AMP, such as fatty acids and bile acids, contribute to the microbicidal activities of meconium, or that pepsin digestion may generate antimicrobial fragments as has been shown for histone H2A (25).

HNP, LL-37, and azurocidin have been shown to be expressed in neutrophils (6,26,27). In addition, LL-37 is expressed in various epithelial cells and specific lymphocytes (28,29). Neutrophil defensins and LL-37 are also present in human colon mucosa, vernix, and amniotic fluid (13,15,19), which make it difficult to establish their original source in meconium.

HD 5 and lysozyme, now identified in meconium and feces, are known to be produced by Paneth cells of the small intestine, and are also present in human breast milk (31–34). HD 5 has here been characterized in material derived from the gastrointestinal tract of the fetus. This suggests that it is produced by the intestine of the fetus in the absence of microbes. This result is supported by Mallow *et al.* (35), showing that HD 5 mRNA is expressed already at 13.5 wk of gestation in the fetal gut. Putsep *et al.* (36) have demonstrated that the cryptdins (defensin-homologues in mice) are expressed under germ-free conditions, suggesting that microbes would not be required for the production of cryptidins. On the other hand, AMP can influence the colonization of the gut, since the composition of the luminal bacteria in the HD 5 transgenic mouse differs compared with the wild-type mouse (37).

LL-37 and lysozyme were present at significantly higher levels in feces than in meconium (Fig. 1*B* and Fig. 5*A*). Lysozyme originating from breast milk is most likely degraded by digestive enzymes in the gastrointestinal tract of the neonate and is most probably not the source of lysozyme in the fecal extracts. Induced expression of AMP in the gut has been shown to be associated with the presence of bacteria and bacterial products (9,10). Thus, our interpretation is that the expression of lysozyme and LL-37 is induced upon bacterial colonization of the neonatal gut. Interestingly, the azurocidin has a different form in meconium, indicating a specific processing step in the fetal gut that is not present after colonization.

The antimicrobial histones (H2A and H4) isolated in this study are cationic proteins usually located in the nucleus where their main function is packing of chromatin. However, histones have been known for their antimicrobial properties for decades and have more recently been linked to innate defenses of the colon (12,13,38,39). Other activities have also been reported for histones, such as hormone-like activity and endotoxic-neutralizing activity in human placenta (40,41). Histone proteins are thus present at extranuclear locations and can be released into the gastrointestinal tract by epithelial cells undergoing apoptosis (39,42). The antimicrobial histones here isolated in meconium may be derived from cells undergoing apoptosis during development (43) and/or turnover of epithelial cells in the fetal intestines, compatible with a role of histones in innate defense.

Our data presented here indicates that colonization affects the level of AMP in the neonatal gut. However, to further elucidate how the innate defense mechanism influences the colonization process, mice models could be used, where different microbicidal peptides and microbial strains of the natural flora of the gut would be monitored at different stages of colonization.

In conclusion, our findings suggest that AMP in meconium and feces act as a defense barrier in the neonatal gut and that this defense system is affected upon colonization after birth.

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