Long-Time Persistence of Superantigen-Producing *Staphylococcus aureus* Strains in the Intestinal Microflora of Healthy Infants

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ABSTRACT

Staphylococcus aureus has been isolated at an increasing rate from infants' stools during the last decades, but it is not known whether this species can colonize and persist in the intestinal microflora. To investigate this, 49 Swedish infants were followed prospectively from birth until 12 months of age. *S. aureus* was identified in a rectal swab obtained 3 d after delivery and in quantitative cultures of fecal samples collected at 1, 2, 4, and 8 weeks and at 6 and 12 months of age. A random amplified polymorphic DNA (RAPD) method was developed to distinguish individual *S. aureus* strains from one another and the strains were tested for production of enterotoxins A-D and TSST-1. By 3 days of age, 16% of infants had *S. aureus* in their intestines, which increased to 73% by 2-6 months, whereafter it decreased slightly to 53%. At the same time *S. aureus* population counts in colonized infants declined from an average $10^{6.8}$ CFU/g feces during the first months of life to $10^{4.0}$ CFU/g feces by 12 months. Colonized infants usually harbored one or two *S. aureus* strains in their microflora for long periods of time. Few strains were transient passengers and the median time of persistence of *S. aureus* strains in the microflora was several months. Of the 75 *S. aureus* strains identified, 43% produced one or more toxins: 13% SEA, 7% SEB, 23% SEC, 4% SED, and 11% TSST-1. Altogether, 47% of the investigated infants were colonized by a toxin-producing *S. aureus* during their first year of life. Despite this they were apparently healthy and did not have more gastrointestinal problems than noncolonized infants. This report is the first to show that *S. aureus* may be a resident member of the normal intestinal microflora in infancy. (*Pediatr Res* **48:** 741–747, 2000)

Abbreviations
CFU, colony-forming units
TSST-1, toxic shock syndrome toxin-1
RAPD, random amplified polymorphic DNA

Bacteria start to colonize the skin, respiratory tract, and intestines as soon as the newborn has left the sterile womb. With time, a complex ecosystem develops known as the normal microflora (1). *Staphylococcus aureus* colonizes the anterior nares of more than 50% of young infants (2–5). During the last decades, *S. aureus* has also been isolated at an increasing rate from fecal samples of infants in Western societies. Thus, only 1 out of 13 infants had *S. aureus* in their stools in a study performed in England in the 1980s (6), but 30% in a Swedish study published 1985 (7) and 53% in Sweden in the late 1990s (8). It is not known whether staphylococci persist and replicate in the intestines or if *S. aureus* in the stools represent bacteria transiently passing the intestines after having been swallowed.

If infants are colonized by *S. aureus* in their intestines, it may have medical consequences. *S. aureus* strains can produce a range of different exotoxins, among them the enterotoxins, which cause vomiting with or without diarrhea and are responsible for staphylococcal food poisoning (9). The enterotoxins, together with toxic shock syndrome toxin-1, also function as "superantigens." By binding to both the Vβ chain of T cell receptors and to MHC class II molecules on antigen presenting cells they activate a broad range of T cells in an antigen nonspecific manner, resulting in massive cytokine secretion (9). Superantigen-producing *S. aureus* cause toxic shock syndrome, which was first recognized in children (10) and later in tampon-using women (11). *S. aureus* toxins with superantigen function have also been implicated in atopic dermatitis (12), sudden infant death (13, 14), and Kawasaki disease (15, 16) although the evidence is circumstantial. Due to their strong effects on the immune system, superantigen-producing *S. aureus* could theoretically play a role in the shaping of the infant's lymphoid system. In this context, it is interesting to note that the rising incidence of allergies in Western countries

has been suggested to be linked to an altered intestinal colonization pattern (17, 18).

Intestinal colonization of an individual may be assessed by quantitative cultures of fecal samples and by determination of persistence of individual bacterial strains in the microflora. High numbers of fecal bacteria are not likely to be the result of contamination from skin flora or ingestion of bacteria. A strain which is present in the intestinal microflora of an individual for at least three weeks can be assumed to have colonized that individual, because a nonreplicating agent would have been eliminated during this time period (19). Thus, to determine whether a bacterial species represents a true colonizer of the intestinal tract or not, individual strains of the species must be distinguished from one another.

S. aureus strains have been typed using restriction fragment length polymorphism (RFLP) and pulse field gel-electrophoresis (PFGE) (20), methods that are time consuming and expensive, and by phage typing that has limited discriminatory capacity. Random amplified polymorphic DNA assay (RAPD) is a PCR-based method that in principle can be applied to all bacterial species (21). No prior knowledge of the gene sequence is needed. Short primers of arbitrary sequence are allowed to bind to bacterial DNA under low stringency conditions. By PCR, a series of DNA fragments of varying length are generated, the pattern of which form a "fingerprint" of a strain (21).

The aim of the study was to determine colonization rates and population counts of *S. aureus* in a group of Swedish infants followed prospectively from birth to 1 year of age. A RAPD method was developed to enable identification of individual strains of *S. aureus* in the microflora and thereby to assess their persistence. We also determined whether these *S. aureus* strains produced enterotoxins and/or TSST-1.

MATERIALS AND METHODS

Infants. Forty-nine healthy Swedish infants born in 1998–99 at Mölndal Hospital in Sweden (3500 deliveries/y) were studied. These children formed part of a prospective birth-cohort study aiming to investigate the relation between intestinal colonization pattern in infancy and later allergy development. Thirty-nine of the infants had at least one parent with atopy, while 10 infants had no allergic parent. In addition to regular sampling of the rectal flora, the feeding pattern and health status of the infants were continuously registered by the parents on questionnaires kept in their homes. The records were checked by a study nurse who interviewed the parents by telephone when the infants were 6 and 12 months old. The study was approved by the Medical Ethics Committee of Göteborg University.

All infants but five were vaginally delivered. Mothers and infants stayed in the maternity ward for 2–3 d, where rooming-in was practiced. All infants were breast-fed from birth. At 1 month of age 80% were still exclusively breast-fed, and by 4 months, 73%. By six months all infants received additional feeds, but partial breast-feeding was still practiced by 67%.

Sampling and analysis of intestinal microflora. A rectal swab was obtained 3 d after delivery. The swab was streaked on agar selective for staphylococci (*Staphylococcus* medium No. 110) and cultured aerobically at 37°C for 2 d to determine the presence of staphylococci. *S. aureus* was identified as specified below.

Quantitative cultures of the fecal flora were performed at 1, 2, 4, and 8 wk and at 6 and 12 months of age. Feces was collected by the parents and put in a sterile Petri dish. The dish was placed in a gas-tight cachet together with an anaerobic generator (Becton Dickinson, Labfab, Sweden), an anaerobic indicator (Oxoid, Hampshire, England) and a damp sponge. The bag was kept refrigerated until being transported to the laboratory, where it was processed within 24 h after collection. Extensive preliminary experiments were performed to ensure that the procedure described above yielded the same results as immediate processing of fecal samples. No alternations in numbers of any of the important groups of aerobic or anaerobic bacteria could be demonstrated during the 24-storage period using this protocol.

Fecal samples were diluted serially in 10-fold steps in peptone-water and cultivated aerobically on *Staphylococcus* medium No. 110 at 37°C for 2 d. The lowest level of detection was 400 CFU (colony forming units)/g feces. Colonies of different morphology were isolated, subcultured on blood agar, Gram-stained and tested for catalase production (22). Isolates with typical Gram-stain appearance and positive catalase test were tested for coagulase production. Coagulase-positive isolates were regarded as *S. aureus* and frozen at [minus]70°C until further analyzed.

Randomly amplified polymorphic DNA (RAPD) assay for typing of S. aureus strains. For development and optimization of the RAPD typing method, 10 different *S. aureus* strains from the culture collection of the University of Göteborg (CCUG numbers: 9,128, 10,777, 10,778, 15,915, 17,621, 25,925, 25,926, 26,215, 32,981, and 32,982) were used.

Bacterial DNA was isolated using the modified Insta Gene protocol (Bio-Rad Hercules, CA, U.S.A.) described by Jayarao *et al.* (23). 1.5 mL of an overnight brain heart infusion broth culture was centrifuged at $17,000 \times g$. The bacteria were washed in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8, Sigma-Aldrich, Stockholm, Sweden) and suspended in 0.2 mL of Insta Gene matrix (Bio-Rad Hercules). The mixture was incubated at 56°C for 30 min followed by 99°C for 10 min and centrifuged at $17000 \times g$ for 5 min. The supernatant DNA concentration was determined by absorbance 260 nm (LKB Biochrom, Ultrospec II, Cambridge, Great Britain).

The following 10-mer oligonucleotide primers were tested: 5'CAGCACCCAC-3' (OPA-13, Kit A, Operon Technologies, Alameda, CA, U.S.A.) (23), 5'-GTGATCGCAG-3'(OPA-10, Kit A, Operon Technologies), 5'-GTGACATGCC-3' (OPE-4, Kit E, Operon Technologies) (23), 5'-AACGGTGACC-3'(OPE-20, Kit E, Operon Technologies) (23), 5'-CCGGCG-GCG-3'(Pharmacia Biotech, Uppsala, Sweden) (24) and 5'-ACGCGCCCT-3' (Pharmacia Biotech, Sweden) (25). Primer OPE-20 was found to give reproducible results and to allow clear distinction between different strains of *S. aureus*.

Amplification of bacterial DNA was performed in PCR-buffer mixture with a total volume of 50 μL, containing optimal amounts of the different reactants which were found to

be: 8 pmol primer (6–12 pmol tested), 10 ng bacterial DNA (10–40 ng tested), 0.2 mmol dNTP (0.1–0.4 mmol tested), 3.5 mmol MgCl$_2$ (1.5–5.5 mmol tested). Distilled water was added to reach a volume of 50 μL. The tube was sealed by a drop of mineral oil and heated to 80°C in a Thermocycler (Perkin Elmer Cetus Model 480, NJ, U.S.A.), whereafter 3U Taq-polymerase (2–3.5 U tested) was added and the temperature risen to 94°C for 120 s for denaturation. 35 PCR cycles were run, each consisting of incubation at 94°C for 70 s, at 33°C for 60 s, and at 72°C for 130 s (23).

The PCR products were separated by PAGE. Ready made 8% Tris-Glycine gels (Novex, Frankfurt, Germany) were applied in a vertical electrophoresis apparatus (Novex). Samples and DNA marker (Boehringer Mannheim, Bromma, Sweden) were loaded on the gel and the electrophoresis was run using Tris-Glycine as running buffer (0.24 M Trisbase, 1.9 M glycine, 0.035 M SDS, pH 8.3) at a constant voltage of 90V for 2 h 15 min. DNA was visualized by silver staining (Plusone DNA silver staining kit, Pharmacia Biotech, Uppsala, Sweden), whereafter the gels were dried (Dry Ease Gel Drying System, Novex).

Toxin production by S. aureus strains. One isolate of each *S. aureus* strain was cultivated overnight in broth and tested for toxin-production by reversed passive latex agglutination. The SET-RPLA kit was used to detect enterotoxin A, B, C, and D and the TST-RPLA kit for TSST-1 (both from Oxoid, Hampshire, Great Britain).

Statistics. Frequencies were compared using Fisher's exact test. Bacterial numbers were compared using Wilcoxon's rank-sum test.

RESULTS

***Colonization frequency and population levels of* S. aureus.** The isolation rate of *S. aureus* in the intestinal microflora of 49 infants is shown in Figure 1. Sixteen percent of the infants were culture-positive for *S. aureus* by day 3. Thereafter, colonization rates increased continuously to reach 73% by 6 mo. By 12 months of age 53% still had *S. aureus* in their stool samples. The decrease in colonization rate between 6 and 12 mo was not quite significant ($p = 0.06$).
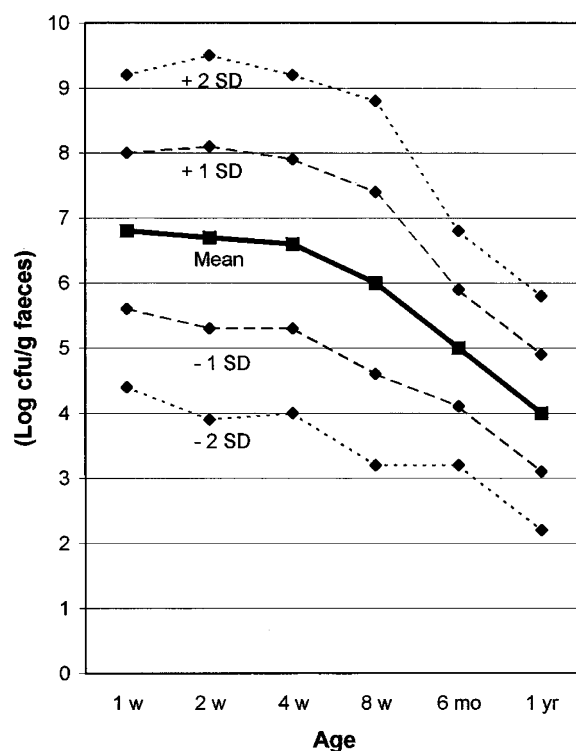
Fecal counts of *S. aureus* in culture positive infants are shown in Figure 2. *S. aureus* population numbers declined continuously throughout the period, from almost 10^7 CFU (colony-forming units) per g feces during the first month to 10^4 CFU/g feces by 1 year of age. The decline in *S. aureus* population numbers was significant between 2 and 6 mo ($p = 0.0004$), as well as between 6 and 12 mo ($p = 0.0001$). There was no difference in *S. aureus* colonization pattern between infants born to atopic or nonatopic parents (data not shown).

Typing of S. aureus isolates obtained from the intestinal flora of infants. The RAPD method for strain typing of *S. aureus* was applied to 262 isolates obtained from the 42 infants who had *S. aureus* in at least one sample.

Figure 3 shows examples of the RAPD patterns of *S. aureus* isolates from three of the infants (children Nos. 1, 5, and 6). Child No. 1 carried two strains during the study period, strain A being represented by seven isolates, and strain B by two
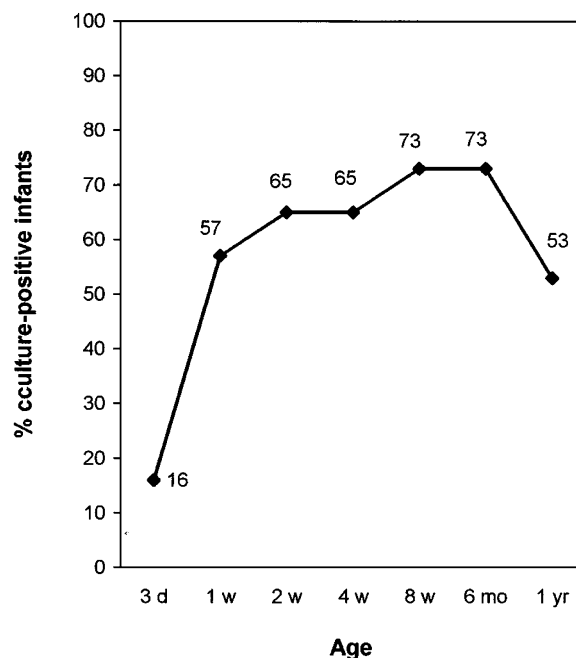

Figure 1. Intestinal *S. aureus* colonization rate in 49 Swedish infants. The 3-day sample consisted of a rectal swab, the rest were fecal cultures in which the limit of detection was 400 CFU/g feces ($10^{2.6}$).

Figure 2. *S. aureus* population counts in fecal samples from culture-positive infants.
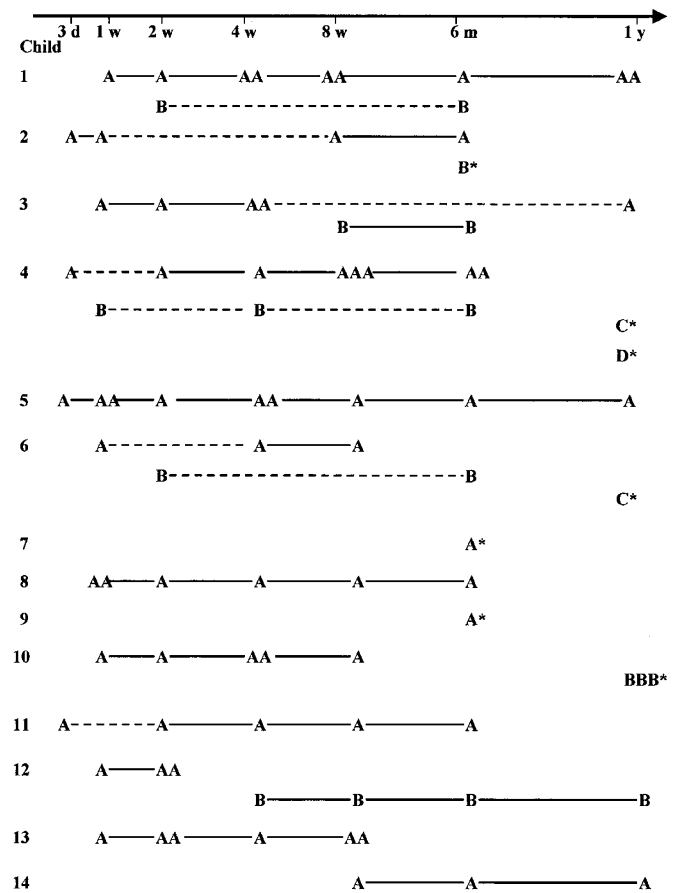
isolates (Fig. 3a). Child 5 carried a single *S. aureus* strain (A) on all occasions (Fig. 3b). Child 6 carried two strains, strain A being represented by three isolates, strain B by a single isolate (Fig. 3b). As evident from the figure, the RAPD patterns could easily be distinguished from one another by the naked eye. Although we did not directly compare RAPD patterns between
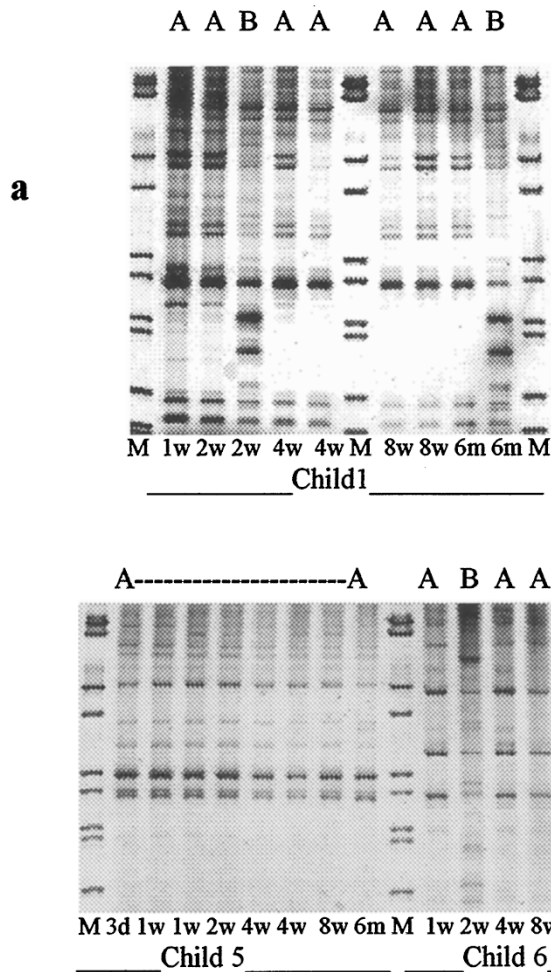
Figure 3. (*A*) and (*B*): RAPD patterns of *S. aureus* isolates from the intestinal microflora of three children. The first strain appearing in a child is termed *A*, the second *B*. M = molecular weight standard. The same children appear also in Fig. 4 (Nos. 1, 5, and 6).

Figure 4. Colonization patterns of 14 children culture positive for *S. aureus* on at least one occasion during their first 12 mo of life. In each infant, the different strains identified are labeled *A*, *B*, *C*, and *D* in their order of appearance. Each letter represents one isolate of the strain. A solid line indicates the continuous presence of the strain in the microflora since it was detected on all culture occasions, a dashed line denotes that the strain was not found on an intervening occasion. An asterisk marks a strain only found once in an infant.

infants, we did not observe two identical RAPD patterns in strains obtained from different infants, suggesting that all identified strains were different. Reproducibility was tested by reanalyzing the same strains by PCR a number of times. The electrophoretic patterns obtained were very similar for the same strain and the strains could always easily be distinguished from one another (data not shown).

The longitudinal *S. aureus* colonization patterns of the first 14 infants is shown in Figure 4. As seen in figure, most of them carried one or two *S. aureus* strains for long periods of time. In several instances, a strain was present in the microflora of an infant, then absent from one or several samples, and then appeared again (a dashed line in figure). Only rarely was a strain present on a single sampling occasion only (strains marked with an asterisk), and in these cases they were found at either the 6 or 12 month sampling occasions, in which case they could have been present for quite some time between the samplings. In the total cohort of 42 culture positive infants, 54% had a single *S. aureus* strain in the microflora during their first 6 months of life, 39% of the infants carried two strains and 7% three strains.

Resident strains were defined as those present in the microflora consecutively for at least 3 weeks. These calculations were made only on the period up to 6 mo, because sampling occurred with too long intervals after this time. Out of the 63 strains identified in 41 infants between 0 and 6 mo age, 15 were of undetermined colonizing capacity, because they were found only in the 6-month sample in which case one cannot know whether the strain would occur at later time-points. Of the remaining 48 strains, 35 (73%) were resident, while only 13 (23%) were transient. The median time of persistence of resident strains was at least 17 wk (range 3w- ≥ 6 mo), the median time of persistence of all strains was 11 wk during this time period.

Of the infants who had *S. aureus* in their microflora by 6 months, one third retained this strain by 12 mo, one third acquired a new *S. aureus* strain between 6 and 12 mo, and another third lost *S. aureus* from their microflora after 6 mo.

Toxin production by intestinal S. aureus strains. One isolate each of the 75 identified *S. aureus* strains was tested for toxin production. Forty-three percent produced one or more toxins: 13% enterotoxin A, 7% enterotoxin B, 23% enterotoxin

C, 4% enterotoxin D, and 11% TSST-1. Twenty-eight percent of the strains produced more than one toxin, most commonly an enterotoxin in combination with TSST-1. Altogether, 23 of the 49 infants (47%) harbored a toxin-producing strain of *S. aureus* in their intestinal microflora during their first 12 months of life.

Toxin production was related to colonization levels and time of persistence in the microflora. Overall, 25% of the toxin producing strains and 16% of the nonproducing strains reached a colonization level of $>10^8$ CFU/g feces (N.S). Fifty-six percent of toxin producing strains were resident, *i.e.* persisted at least 3 wk, compared with 44% of nontoxin producing strains (N.S).

Clinical characteristics of colonized infants. Gastrointestinal problems were reported in nine infants. The symptoms included diarrhea (two infants), colic (three infants), constipation (two infants) and abdominal pain (four infants). All these nine infants were colonized by *S. aureus*, but only three of them by toxin-producing strains (1 SEC, 1 SEC-TSST-1, 1 SED). Thus, we did not detect any overt clinical consequences of intestinal colonization by toxin-producing *S. aureus*.

DISCUSSION

In the present study, we have demonstrated that *S. aureus* has become a major colonizer of the intestinal tract of Swedish infants. More than 80% of the infants studied had *S. aureus* in their stool samples at any time during their first year of life. Between 2 and 6 months of age, when colonization rate was maximal, three children out of four had *S. aureus* in their intestinal microflora at any point in time. Although colonization declined somewhat after 6 months of age, approximately 50% of the infants were still culture-positive by 12 months of age. The possibility that this represented contamination by skin flora during sampling or by swallowed *S. aureus* from nasal secretion was negated by the high population counts (median $10^{6.7}$ CFU/g feces by 1 month of age). Furthermore, contrary to the colonization pattern described here, nasal carriage of *S. aureus* is maximal in the perinatal period and decreases thereafter (3, 4).

The possibility that the bacteria were transient passengers present in the stools, *e.g.* because of intake of contaminated food, was negated by an average persistence of individual *S. aureus* strains in infants' flora of several months. Some *S. aureus* strains seemed to come and go in the microflora of an infant. Such strains were present during one or several samples, then absent to appear again at later time points. These strains might have been suppressed by other bacteria during a period, or they were simply missed during the isolation procedure because of the simultaneous presence in all infants of large numbers of coagulase negative staphylococci, which grew on the same plates. A third possibility is that the child was re-colonized by the same strain, possibly from someone in the family.

Among intestinal bacteria, only *Escherichia coli* has so far been studied with regard to longitudinal colonization patterns of individual strains. Certain strains, termed resident, persist in the microflora for months or years, while others, termed tran-

sient, do not colonize but disappear from the flora in one or a few weeks' time (26–29). Swedish infants often carry a single resident *E. coli* strain in their microflora for many months and the transient strains are few (30). This has been attributed to low environmental exposure to *E. coli*, since infants born in Pakistan exhibit a rapid turnover of individual *E. coli* strains in their microflora (29). The stable *S. aureus* colonization pattern seen here is, thus, not indicative of an overwhelming exposure to this bacterial species. We instead suggest that *S. aureus* is so common in Swedish infants due to lack of competition with other bacterial groups better suited for colonization of the large bowel. Neonatal colonization with *E. coli* and other enterobacteria seems to have declined in Sweden during the last decades, most likely due to a generally increased hygiene and reduced spread of these bacteria in maternity wards (31) (Adlerberth *et al.* in preparation). In accordance, *S. aureus* population levels were quite high during the 1st month, but decreased progressively with time even if colonization continued. We noted a similar decrease in fecal counts of coagulase negative staphylococci with time, while the counts of *Enterobacteriaceae* and enterococci stayed high (unpublished observations). These findings implicate that staphylococci cannot compete favorably with more "traditional" intestinal bacteria when a more complex microflora develops.

We believe that the source of *S. aureus* colonizing the infants was the skin flora of the people in the infants' home milieu. Colonization was low by 3 d' age and increased continuously, which speaks against transfer at delivery or acquisition at the hospital, where these infants stayed only for 2–3 d. Moreover, we have in several instances observed strains with identical RAPD patterns on a mother's skin and in her infant's fecal samples (Lindberg, unpublished results). The very high frequency of long duration breast-feeding in Sweden could contribute to transfer of *S. aureus* from mother to infant. *S. aureus* is a more common colonizer of breast-fed than formula-fed infants, probably because these bacteria colonize the skin around the nipple and are swallowed during suckling (7, 32, 33).

Somewhat less than half of the *S. aureus* strains produced enterotoxin A, B, C, or D, and/or TSST-1, which is in accordance with previous studies that report a rate of toxin production of 30% to 50% in clinical isolates of *S. aureus* (34–36). We found that enterotoxin C was the toxin most commonly produced, followed by enterotoxin A and TSST-1, while enterotoxins B and D were the least common ones. The dominance of enterotoxin C isolates is similar to what has been reported for human skin isolates of *S. aureus* (34, 37).

Enterotoxins induce vomiting and diarrhea and *S. aureus* enterotoxins share with TSST-1 the "superantigen" function. This means that they can activate a broad range of T cells resulting in massive cytokine production, a mechanism responsible for toxic-shock-syndrome (9). Despite this, infants colonized by toxin-producing strains were apparently healthy and did not have more gastrointestinal problems than others. The extent of expression of the toxins *in situ* in the intestine remains to be studied. The temperature in the intestinal lumen is 37°C, which is optimal for toxin production (38). On the other hand, the oxygen content might be too low to enable their

synthesis (38). However, if intestinal *S. aureus* gain entrance into the tissues by translocation across the intestinal barrier, they would most probably be able to produce toxins. Translocation of bacteria is proportional to bacterial population levels in the gut. For example, when *E. coli* population levels exceed 10^8/g faeces, live *E. coli* bacteria can be found in the mesenteric lymph nodes of experimental animals (39, 40). A number of the infants studied here were colonized by *S. aureus* strains reaching such high population levels during their 1st weeks of life.

It is also possible that toxins are produced *in vivo*, but that their action is neutralized by antibodies present in breast-milk (41). Since practically all adults are sero-positive against *S. aureus* toxins and such antibodies are transferred *via* the placenta, anti-toxin antibodies should also be present in most infant sera (42–44). The fact that most infants have been exposed to toxin-producing *S. aureus* is indicated by findings of protective levels against the *S. aureus* toxin TSST-1 in 30% of 2-year-old U.S. children (43).

S. aureus is one of the major causes of neonatal septicaemia in Sweden and other Western countries (45). Septicaemia due to enterobacteria has been suggested to result from direct translocation of the bacteria over the intestinal barrier because the same strain of *E. coli* or other enterobacteria can be isolated from both the blood and the intestinal microflora (46, 47). It is thus conceivable that septic isolates of *S. aureus* may in some cases derive from the intestinal microflora, rather than from the skin as has previously been assumed. In accordance, infants struck by *S. epidermidis* septicaemia often have *S. epidermidis* in their stools (48).

The fact that so many children carry *S. aureus* in their intestinal microflora, and that *S. aureus* strains may persist for several months in their intestines, has not been reported before. A low level of colonization with *E. coli* and maybe other groups of intestinal commensal bacteria has probably paved the way for *S. aureus*, which has previously not been recognized as a commensal intestinal species. Since this pattern of colonization is likely to have evolved during the last decades, the consequences for infant health remains to be studied.

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