Polyclonality of *Staphylococcus epidermidis* residing on the healthy ocular surface

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Staphylococcus epidermidis is part of the normal bacterial flora on the ocular surface. The chromosomal DNA of bacterial isolates obtained from the conjunctival sac, upper and lower lid margins, and upper and lower Meibomian glands of healthy volunteers was subjected to *Sma*I digestion and PFGE to study the genetic diversity of the organisms. Multiple colonies were also examined of *S. epidermidis* derived from the conjunctival sac of the same subjects. Lastly, commensal bacteria were harvested from the ocular surfaces of four healthy subjects once a month for 6 months, and the genetic background of the *S. epidermidis* isolates was analysed. It was found that bacterial strains not only from different subjects but also from multiple ocular surface sites of the same subject exhibited different PFGE patterns. In five of 42 subjects multiple colonies of *S. epidermidis* were isolated from the conjunctival sac; three harboured multiple colonies with different PFGE patterns, and two manifested multiple colonies with identical PFGE patterns. *S. epidermidis* isolated from the conjunctival sac of the same subjects over a 6-month period exhibited varying PFGE patterns. The data demonstrate the polyclonality of *S. epidermidis* on the healthy ocular surface.

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INTRODUCTION

In humans, the largest populations of staphylococci are usually found in areas of the skin and mucous membranes surrounding body openings. Some staphylococcus species exhibit a marked preference for certain habitats; in adults, the coagulase-positive species *Staphylococcus aureus* prefers the anterior nares. On the other hand, the predominant staphylococcus species *Staphylococcus epidermidis* is widely distributed over the body surface (Kloos & Musselwhite, 1975).

Bacterial flora composed of *S. epidermidis*, *Propionibacterium acnes*, and other Gram-positive and -negative bacteria (Doyle *et al.*, 1995; Hara *et al.*, 1997), is normal in the conjunctival sac and eyelid edge, on skin, and in mucosal tissues. *S. epidermidis* is representative of the normal bacterial flora on the ocular surface and is most frequently isolated from the microflora of the human ocular surface.

We subjected *S. epidermidis* strains isolated from healthy ocular surfaces to PFGE to study the diversity in the genetic background of this bacterium. We show that *S. epidermidis* on the human ocular surface can be of polyclonal origin.

METHODS

All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants and their prior written informed consent was obtained.

S. epidermidis

Analysis of bacterial isolates from multiple ocular surface sites of the same subject. *S. epidermidis* was obtained from five sites on the healthy ocular surface of volunteers, i.e. the conjunctival sac, upper and lower lid margins, and upper and lower Meibomian glands, using CultureSwab (Becton Dickinson). The isolates obtained were stored (–20 °C) in ANAport BIKEN culture medium (BIKEN) at the Department of Ophthalmology of Kyoto Prefectural University of Medicine; they were sent to The Research Foundation for Microbial Diseases of Osaka University on the next day.

Analysis of multiple colonies obtained from the conjunctival sac of the same subject. Commensal bacteria were obtained from the conjunctival sac of volunteers with no ocular diseases using CultureSwab at The Research Foundation for Microbial Diseases of Osaka University, and the isolates were identified without being stored in order to detect bacteria more efficiently. Multiple colonies of *S. epidermidis* derived from conjunctival sacs of the same subjects were subjected to PFGE.

Chronological analysis of bacterial changes occurring in the conjunctival sac of the same subject over a 6-month period.

Commensal bacteria in the conjunctival sacs of four healthy volunteers were obtained once a month for 6 months using CultureSwab, and the genetic background of *S. epidermidis* was analysed by PFGE.

All isolates were identified at The Research Foundation for Microbial Diseases of Osaka University. The isolates were cultured by both direct culture and enrichment culture. In direct-culture methods, bacteria were isolated on Columbia CNA agar with 5% sheep blood (Becton Dickinson) at 37 °C for 48 h aerobically, or for 5 days anaerobically. In enrichment-culture methods, isolates were cultured in thioglycolate medium (Eiken Chemical) at 37 °C for 1–2 weeks, and if positive for bacteria, the bacteria were isolated on Columbia CNA agar with 5% sheep blood at 37 °C for 48 h aerobically or for 5 days anaerobically. *S. epidermidis* was identified using the API 20 Staph system (bioMérieux).

PFGE. For genotyping, we performed PFGE analysis of bacterial chromosomal DNA after *Sma*I digestion, because this is the most highly discriminating method for coagulase-negative staphylococci (Linhardt *et al.*, 1992). The GenePath System (Bio-Rad Laboratories) was used for genetic fingerprinting. Isolates cultured overnight in brain heart infusion broth (Difco) were subjected to *Sma*I digestion of bacterial genomic DNA according to the manufacturer's instructions (GenePath Group I Reagent kit; Bio-Rad). PFGE patterns were obtained by separating the digested DNA on 1% agarose gels (molecular biology certified agarose, Bio-Rad) in a counter-clamped homogeneous CHEF-DR Mapper (GenePath system, Bio-Rad). The electrophoresis buffer was 0.5 × TBE buffer (45 mM Tris/borate, 1 mM EDTA); equilibration was at 14 °C using a constant voltage of 6 V cm⁻¹ with pulse-ramping from 5.3 to 34.9 s for 20 h at a 120° angle. A lambda ladder consisting of successively larger concatemers of 48.5 kb DNA fragments was used as the molecular size marker. Electrophoresed gels were stained with ethidium bromide, washed with distilled water, and photographed under UV light.

DNA bands were compared by visual inspection and interpreted according to Tenover *et al.* (1995). Based on the number of DNA fragments that exhibited different pulse patterns, the strains were

considered to be indistinguishable (identical pulse patterns), closely related (two to three fragments with different patterns), or possibly related (four to six fragments with different patterns). If more than six DNA fragments exhibited a different migration pattern, the isolates were considered to be unrelated.

RESULTS AND DISCUSSION

We first analysed commensal bacteria isolated from the conjunctival sac, upper and lower lid margins, and upper and lower Meibomian glands of healthy volunteers. *S. epidermidis* was isolated from 12 of 40 volunteers sampled at Kyoto Prefectural University of Medicine; seven of them harboured *S. epidermidis* at multiple ocular surface sites. Strains obtained from different subjects exhibited different and unrelated PFGE patterns. *S. epidermidis* isolated from multiple sites in a single subject did not exhibit monoclonal PFGE patterns (Fig. 1). Our results indicated that the ocular surface of healthy individuals can harbour polyclonal *S. epidermidis*.

We next harvested commensal bacteria from conjunctival sacs of 42 healthy volunteers at The Research Foundation for Microbial Diseases of Osaka University. The isolated commensal bacteria are listed in Table 1. Bacteria were isolated from the conjunctival sac of 29 of 42 volunteers (Table 1). *S. epidermidis* was isolated from 19 of 42 volunteers (Table 2). *S. epidermidis* was mainly isolated aerobically. Of these 19 subjects, five harboured multiple colonies of *S. epidermidis*, as determined by direct or enrichment culture (Table 1, volunteers 1, 2, 5, 27 and 29) and three of these five subjects harboured multiple colonies that manifested different and closely related, possibly

Fig. 1. *S. epidermidis* isolated from multiple sites on the healthy ocular surface. *S. epidermidis* isolated from five sites on the healthy ocular surface (conjunctival sac, upper and lower lid margins, upper and lower Meibomian glands) were analysed by PFGE (C, conjunctiva; UL, upper lid margin; LL, lower lid margin; UM, upper Meibomian gland; LM, lower Meibomian gland). The PFGE patterns of *S. epidermidis* isolated from multiple sites of the same subject were different but closely related or possibly related. Marker, molecular mass marker of concatemers of lambda phage DNA. (a–d) show *S. epidermidis* isolated from different subjects.

Table 1. Commensal bacteria isolated in this study

Volunteer	Direct culture				Enrichment culture	
	Aerobe	No. of isolates	Anaerobe	No. of isolates	Aerobe and anaerobe	No. of isolates
1	<i>S. epidermidis</i>	11	<i>P. acnes</i>	7	<i>S. epidermidis</i>	2
2	<i>S. epidermidis</i>	3	<i>P. acnes</i>	4	<i>S. epidermidis</i>	1
			<i>Propionibacterium granulosum</i>	1		
3		0		0	<i>P. acnes</i>	1
4	<i>S. epidermidis</i>	1		0	<i>P. acnes</i>	1
5	<i>S. epidermidis</i>	35	<i>S. epidermidis</i>	26	<i>S. epidermidis</i>	1
			<i>P. acnes</i>	23		
6		0	<i>P. acnes</i>	9	<i>P. acnes</i>	1
7		0	<i>P. acnes</i>	5	<i>S. epidermidis</i>	1
					<i>Staphylococcus hominis</i>	1
8		0		0	<i>S. epidermidis</i>	1
9		0		0	<i>Staphylococcus warneri</i>	1
10		0		0	<i>S. epidermidis</i>	1
11		0		0	<i>S. epidermidis</i>	1
12		0		0	<i>P. acnes</i>	1
13		0		0	<i>Staphylococcus lugdunensis</i>	1
14		0		0	<i>S. epidermidis</i>	1
15		0		0	<i>S. epidermidis</i>	1
16		0		0	<i>S. epidermidis</i>	1
17		0		0	<i>Staphylococcus intermedius</i>	1
18		0		0	<i>S. epidermidis</i>	1
19		0	<i>P. acnes</i>	2	<i>S. epidermidis</i>	1
20		0		0	<i>Alteromonas denitrificans</i>	1
21		0		0	<i>S. intermedius</i>	1
22		0	<i>P. acnes</i>	1	<i>P. acnes</i>	1
23		0	<i>P. acnes</i>	1		0
24		0		0	<i>S. epidermidis</i>	1
25		0		0	<i>S. epidermidis</i>	1
26		0	<i>P. acnes</i>	1	<i>S. epidermidis</i>	1
27	<i>S. epidermidis</i>	2	<i>P. acnes</i>	1	<i>S. epidermidis</i>	1
28		0		0	<i>S. epidermidis</i>	1
29	<i>S. epidermidis</i>	1		0	<i>S. epidermidis</i>	2
					<i>S. lugdunensis</i>	1

related, or unrelated PFGE patterns (Fig. 2a, b). The other two subjects were host to multiple colonies with identical and indistinguishable PFGE patterns (Fig. 2c). These results indicate that polyclonal *S. epidermidis* may reside not only on the ocular surface, but also in the conjunctiva.

Lastly, we analysed commensal bacteria harvested once a month for 6 months from the conjunctival sac of four

Table 2. Isolation of commensal bacteria from conjunctival sacs of 42 healthy volunteers

Organism	No. of isolates	As a percentage
<i>S. epidermidis</i>	19/42	45 %
<i>P. acnes</i>	13/42	31 %
Others	8/42	19 %
Not detected	13/42	31 %

healthy volunteers (Table 3). We found that although the make-up of the microbial populations changed slightly over the course of this investigation, *S. epidermidis* and *P. acnes* remained dominant. In three out of the four subjects, we detected *S. epidermidis* at multiple sampling.

We analysed *S. epidermidis* isolated from the conjunctival sac of the same subject at different time points and found that the organisms manifested variously related or unrelated PFGE patterns. Although these patterns remained relatively similar, the *S. epidermidis* organisms were polyclonal and yielded multiple colonies with unrelated PFGE patterns over the course of our study. At some sampling points there was also a change in the dominant strain (Fig. 3).

To the best of our knowledge, this is the first PFGE investigation to examine the diversity in the genetic background of *S. epidermidis* residing on the healthy human ocular surface. Strains of *S. epidermidis* harvested

Fig. 2. Multiple colonies of *S. epidermidis* obtained from the conjunctival sac of the same subject. (a) Volunteer 1, six of 11 *S. epidermidis* colonies grown by direct culture. (A) and (B) are unrelated; (B) and (C) are closely related. (b) Volunteer 29, one colony was grown in direct culture and the other two in enriched culture. (D) and (E) are unrelated; (F) is unrelated to (D) and (E). (c) Volunteer 2, three colonies were grown in direct culture and one was grown in enriched culture. All were indistinguishable. Marker, molecular mass marker of concatamers of lambda phage DNA.

from different ocular surface sites of the same donor manifested similar, albeit distinct, PFGE patterns. In five of 19 subjects with *S. epidermidis* in their conjunctival sacs, direct or enriched cultures grew multiple colonies; the PFGE patterns of each colony were similar but distinct in three, and identical in the other two. These results suggest that *S. epidermidis* inhabiting not only the ocular surface, but also the conjunctiva, are polyclonal. The harvest of *S. epidermidis* from the conjunctival sac of the same subject at different times revealed that while the PFGE patterns of isolates remained relatively similar over time, the organisms were polyclonal and yielded multiple colonies with unrelated PFGE patterns. A change in the dominant strain was also suggested at some sampling times. These observations confirm the polyclonality of *S. epidermidis* on the healthy ocular surface.

The largest populations of human staphylococci reside in areas of the skin and mucous membranes that surround openings in the body surface. Some *Staphylococcus* species

exhibit a marked preference for certain habitats; for example, in adults, coagulase-positive *S. aureus* preferentially inhabits the anterior nares, and the predominant *S. epidermidis* species is widely distributed over the body surface (Kloos & Musselwhite, 1975).

Bacterial flora composed of *S. epidermidis*, *P. acnes*, and other Gram-positive and Gram-negative bacteria, is normally found in the conjunctival sac or on the eyelid edge (Doyle *et al.*, 1995; Hara *et al.*, 1997). *S. epidermidis* is representative of the normal bacterial flora, and is most frequently isolated from the microflora of the human ocular surface.

Hu *et al.* (1995a) have reported that *S. epidermidis* organisms isolated from the nares of healthy subjects are polyclonal, while *S. aureus* inhabiting the nares of the same subjects is monoclonal (Hu *et al.*, 1995b). Similarly, our results revealed that *S. epidermidis* existing as normal bacterial flora on the healthy ocular surface is polyclonal. Of 40 subjects that we

Table 3. Commensal bacteria harvested over a 6-month period from the conjunctival sac of four healthy volunteers

d, direct culture; e, enrichment culture; the number of colonies is given after d or e.

Month*	Volunteer 1	Volunteer 2	Volunteer 3	Volunteer 4
1	<i>Propionibacterium avidum</i> (e1)	<i>S. epidermidis</i> (e1)	<i>S. epidermidis</i> (e1)	<i>S. epidermidis</i> (e1)
2	—	<i>S. epidermidis</i> (e2)	<i>P. acnes</i> (d1)	<i>P. acnes</i> (e1)
3	<i>P. acnes</i> (e1)	<i>P. acnes</i> (d6)	<i>S. epidermidis</i> (d3, e1)	<i>S. epidermidis</i> (e1)
4	—	<i>P. granulosum</i> (d1)	<i>P. acnes</i> (d4)	<i>S. epidermidis</i> (d1)
5	<i>P. acnes</i> (e1)	<i>S. epidermidis</i> (d1, e2)	<i>S. epidermidis</i> (e2)	<i>P. acnes</i> (e1)
6	—	<i>S. epidermidis</i> (e1)	<i>S. epidermidis</i> (e1)	—
7	<i>P. acnes</i> (e1)	<i>S. epidermidis</i> (e1)	<i>P. acnes</i> (d21)	—
8	<i>S. epidermidis</i> (e1)	<i>P. acnes</i> (d5)	<i>S. aureus</i> (e1)	—
9	—	<i>S. hominis</i> (e1)	<i>S. epidermidis</i> (e1)	—

*The month in which the samples were collected.

Fig. 3. *S. epidermidis* harvested at different times from the conjunctival sac of the same subjects. (a) A 42-year-old woman. Of eight colonies, one was grown in direct and seven in enriched culture. At four sampling times (months 1, 2, 3 and 4), the same clone (A) of *S. epidermidis* was detected. At 2- and 3-month sampling, two clones (A and B) of *S. epidermidis* were detected. Sampling on the fifth month detected one clone (C) of *S. epidermidis*; it was closely related to (A). (b) A 36-year-old man. Of nine colonies, three were grown in direct and six in enriched culture. At 1- and 2-month sampling, the same clone (D) of *S. epidermidis* was detected. At 3- and 4-month sampling, clone (E) was detected. It was unrelated to (D). In the sixth month, clone (F), unrelated to (E), was detected. M, molecular mass marker of concatemers of lambda phage DNA.

examined, two harboured *S. aureus* at multiple ocular sites. The PFGE patterns of *S. aureus* isolated from multiple ocular sites of a single subject were identical and indistinguishable (data not shown), supporting the hypothesis that *S. aureus* organisms residing in the human nares and ocular surface are monoclonal. In contrast to *S. epidermidis*, which is present in the nares of almost all healthy individuals, *S. aureus* is found in the nares of only about 30 % of healthy subjects (Hu *et al.*, 1995a). According to Doyle *et al.* (1995), only 6 % of healthy subjects harbour *S. aureus* on the ocular surface, while 90 % host *S. epidermidis*. As *S. aureus* is an ophthalmic pathogen that can induce bacterial conjunctivitis, keratitis and blepharitis (Kupferman & Leibowitz, 1977; McCulley *et al.*, 1982; Prentice *et al.*, 1977), it may harbour pathogenicity even when present in the nares of healthy individuals.

Due to its ubiquity and relatively low virulence, *S. epidermidis* has been considered as a clinically insignificant contaminant when isolated from clinical samples (Eng *et al.*, 1982). However, *S. epidermidis* is increasingly recognized as an important agent of nosocomial infection (Kloos & Bannerman, 1994; Pfaller & Herwaldt, 1988), and many of these infections have been traced to monoclonal *S. epidermidis* (Burnie *et al.*, 1997; Murono *et al.*, 2003; Nouwen *et al.*, 1998). In the majority of immunocompromised patients, the causative organisms of bloodstream infections originate primarily from the patients' own flora (Kloos & Bannerman, 1994; Murono *et al.*, 2003).

Although *S. epidermidis* is a common component of the normal ocular flora, it can lead to chronic blepharitis, conjunctivitis and keratitis, especially in immunocompromised hosts (Baum, 1978; Pinna *et al.*, 1999), suggesting that opportunistic infection with *S. epidermidis* is reflective of the status of the host. We encountered one instance in which monoclonal *S. epidermidis* was isolated from multiple sites

in both eyes (data not shown). The host was an immunocompromised patient who had undergone bone marrow transplantation and developed superficial punctate keratitis (SPK). Disruption of the balance between *S. epidermidis*, which is a commensal bacterium on the ocular surface, and the status of this host, may have resulted in the monoclonality of the bacteria. We postulate that a good balance between commensal bacteria and hosts maintains the polyclonality of *S. epidermidis* and that a weakened host status may contribute to its pathogenicity. Studies are under way in our laboratories to examine this issue.

In summary, we examined the diverse genetic background of *S. epidermidis* isolated from the ocular surface of healthy subjects, and found that the bacterium may be polyclonal on healthy ocular surfaces.

REFERENCES

- Baum, J. L. (1978). Current concepts in ophthalmology. Ocular infections. *N Engl J Med* **299**, 28–31.
- Burnie, J. P., Naderi-Nasab, M. K., Loudon, W. & Matthews, R. C. (1997). An epidemiological study of blood culture isolates of coagulase-negative staphylococci demonstrating hospital-acquired infection. *J Clin Microbiol* **35**, 1746–1750.
- Doyle, A., Beigi, B., Early, A., Blake, A., Eustace, P. & Hone, R. (1995). Adherence of bacteria to intraocular lenses: a prospective study. *Br J Ophthalmol* **79**, 347–349.
- Eng, R. H., Wang, C., Person, A., Kiehn, T. E. & Armstrong, D. (1982). Species identification of coagulase-negative staphylococcal isolates from blood cultures. *J Clin Microbiol* **15**, 439–442.
- Hara, J., Yasuda, F. & Higashitsutsumi, M. (1997). Preoperative disinfection of the conjunctival sac in cataract surgery. *Ophthalmologica* **211** (Suppl. 1), 62–67.
- Hu, L., Umeda, A. & Amako, K. (1995a). Typing of *Staphylococcus epidermidis* colonizing in human nares by pulsed-field gel electrophoresis. *Microbiol Immunol* **39**, 315–319.

- Hu, L., Umeda, A., Kondo, S. & Amako, K. (1995b).** Typing of *Staphylococcus aureus* colonising human nasal carriers by pulsed-field gel electrophoresis. *J Med Microbiol* **42**, 127–132.
- Kloos, W. E. & Bannerman, T. L. (1994).** Update on clinical significance of coagulase-negative staphylococci. *Clin Microbiol Rev* **7**, 117–140.
- Kloos, W. E. & Musselwhite, M. S. (1975).** Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. *Appl Microbiol* **30**, 381–385.
- Kupferman, A. & Leibowitz, H. M. (1977).** Topical antibiotic therapy of staphylococcal keratitis. *Arch Ophthalmol* **95**, 1634–1637.
- Linhardt, F., Ziebuhr, W., Meyer, P., Witte, W. & Hacker, J. (1992).** Pulsed-field gel electrophoresis of genomic restriction fragments as a tool for the epidemiological analysis of *Staphylococcus aureus* and coagulase-negative staphylococci. *FEMS Microbiol Lett* **74**, 181–185.
- McCulley, J. P., Dougherty, J. M. & Deneau, D. G. (1982).** Classification of chronic blepharitis. *Ophthalmology* **89**, 1173–1180.
- Murono, K., Hirano, Y., Koyano, S., Ito, K. & Fujieda, K. (2003).** Molecular comparison of bacterial isolates from blood with strains colonizing pharynx and intestine in immunocompromised patients with sepsis. *J Med Microbiol* **52**, 527–530.
- Nouwen, J. L., van Belkum, A., de Marie, S., Sluijs, J., Wielenga, J. J., Kluytmans, J. A. & Verbrugh, H. A. (1998).** Clonal expansion of *Staphylococcus epidermidis* strains causing Hickman catheter-related infections in a hemato-oncologic department. *J Clin Microbiol* **36**, 2696–2702.
- Pfaller, M. A. & Herwaldt, L. A. (1988).** Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. *Clin Microbiol Rev* **1**, 281–299.
- Pinna, A., Zanetti, S., Sotgiu, M., Sechi, L. A., Fadda, G. & Carta, F. (1999).** Identification and antibiotic susceptibility of coagulase negative staphylococci isolated in corneal/external infections. *Br J Ophthalmol* **83**, 771–773.
- Prentice, M. J., Hutchinson, G. R. & Taylor-Robinson, D. (1977).** A microbiological study of neonatal conjunctivae and conjunctivitis. *Br J Ophthalmol* **61**, 601–607.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995).** Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.