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Assessment of bacterial contamination of lipstick using pyrosequencing

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Synopsis

As soon as they are exposed to the environment, cosmetics become contaminated with microorganisms, and this contamination accumulates with increased use. In this study, we employed pyrosequencing to investigate the diversity of bacteria found on lipstick. Bacterial DNA was extracted from 20 lipstick samples and mixed in equal ratios for pyrosequencing analysis. As a result, 105 bacterial genera were detected, four of which (*Leifsonia*, *Methylobacterium*, *Streptococcus*, and *Haemophilus*) were predominant in 92% of the 19,863 total sequence reads. Potentially pathogenic genera such as *Staphylococcus*, *Pseudomonas*, *Escherichia*, *Salmonella*, *Corynebacterium*, *Mycobacterium*, and *Neisseria* accounted for 27.6% of the 105 genera. The most commonly identified oral bacteria belonged to the *Streptococcus* genus, although other oral genera such as *Actinomyces*, *Fusobacterium*, *Porphyromonas*, and *Lactobacillus* were also detected.

INTRODUCTION

Cosmetics should be free from pathogenic microorganisms while also maintaining a low count of aerobic microorganisms (1). Microorganism-contaminated cosmetics can affect product quality and pose serious consumer health risks if the contaminating agents are pathogenic (1,2). In general, bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Candida albicans*, *Clostridium* spp., and *Pseudomonas aeruginosa* should be absent (3). Lipstick is at risk of contamination by airborne microorganisms and saliva from the moment it is opened and contacts the lips and skin, a risk that only increases with use (4).

Although diverse bacterial species have been isolated from lipstick, most studies to date have been limited to using counts from culturable aerobic bacteria (3–6). In recent years, however, it has become possible to detect the presence of unculturable bacteria using pyrosequencing. In this study, we used pyrosequencing to examine the diversity of contaminating bacteria in 20 lipstick samples.

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MATERIALS AND METHODS

SAMPLING

Twenty lipstick samples used by women between the ages of 20 and 50 were tested for microbial contamination. Before opening the sample cap, the lipstick surface was cleaned with 70% ethanol. The surface of the lipstick was then swabbed using a sterile cotton swab. Samples of the swab surface were suspended in 1 ml of sterile distilled water.

GENOMIC DNA EXTRACTION

For extraction of bacterial DNA from each of the 20 lipstick samples, G-spin Genomic DNA Extraction Kit for bacteria (Intron Biotechnology Inc., Seongnam-si, Korea) was used, and DNA was extracted according to the manufacturer's instructions. Pyrosequencing analysis was performed once with the target sample consisting of the 20 DNA samples mixed in equal ratios.

PYROSEQUENCING

Polymerase chain reaction (PCR) amplification was performed using primers targeting the V3 to V4 regions of the 16S rRNA gene found in the extracted DNA. For bacterial amplification, primers 341F (5′-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′; underlining sequence indicates the complimentary region of the primer) and 805R (5′-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′) were used. The amplifications were carried out under the following conditions: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final elongation at 72°C for 5 min. Secondary amplification for attaching the Illumina NexTera barcode was performed with i5 forward primer (5′-AAT-GATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-3′; X indicates the barcode region) and i7 reverse primer (5′-CAAGCAGAAGACGGCATAC-GAGAT-XXXXXXXX-AGTCTCGTGGGCTCGG-3′). Conditions for secondary amplification were similar to the previous one except with eight cycles of amplification. The correct PCR product was confirmed using electrophoresis on a 2% agarose gel followed by visualization under a Gel Doc system (BioRad, Hercules, CA). The amplified products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Equal concentrations of purified products were pooled together and short fragments corresponding to nontarget products were removed with the Ampure bead kit (Agencourt Bioscience, Beverly, MA). Sample quality and product size were assessed using a Bioanalyzer 2100 (Agilent, Palo Alto, CA) and a DNA 7500 chip (Agilent). Mixed amplicons were pooled and the sequencing was carried out at Chunlab, Inc. (Seoul, Korea) using an Illumina MiSeq Sequencing system (Illumina, San Diego, CA) according to the manufacturer's instructions.

SEQUENCING DATA ANALYSIS

Obtained reads were sorted using the unique barcodes of each PCR product. The barcode, linker, and primer sequences were removed from the original reads. Any reads containing

two or more ambiguous nucleotides, a low-quality score (average score < 25), or reads shorter than 300 bp were discarded. Potential chimerical sequences were detected using the Bellerophon method consisting of comparing the BLASTN search results between the forward and reverse half sequences (7). After removing chimerical sequences, the taxonomic classification of each read was assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (8). Briefly, this database contains the 16S rRNA gene sequences of type strains along with valid published names and representative species phylotypes of cultured and uncultured entries in the GenBank database. Complete hierarchical taxonomic classifications from phylum to species are also included.

PATHOGENIC BACTERIA ANALYSIS

Cases within the last 10 year reporting the pathogenicity of each identified bacterial genera identified were searched for using PubMed. Pyrosequencing results were matched to the genus level, with the limitation that the analysis could not be completed up to the species level. Therefore, a genus was considered pathogenic when any one of the species belonging to it had a reported case of pathogenicity.

RESULTS

A total of 19,863 sequence reads were obtained and 105 genera of bacteria were identified (Table I). The bacteria identified included those found not only on the skin, but also in saliva and water. *Leifsonia* (65.86%), *Methylobacterium* (14.95%), *Streptococcus* (7.51%), and *Haemophilus* (3.58%) were predominant among all identified genera (Figure 1). Pathogenic bacteria such as *Staphylococcus*, *Pseudomonas*, *Escherichia*, *Salmonella*, *Corynebacterium*, *Mycobacterium*, and *Neisseria* were also found. These potentially pathogenic bacteria represented 27.6% of the 105 genera, whereas the four most dominant genera comprised 92% of the 19,863 total reads. *Actinomyces*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Lactobacillus*, *Streptococcus*, *Veillonella*, and *Fusobacterium* were among the oral bacteria identified. The most commonly identified oral bacteria belonged to the *Streptococcus* genus.

DISCUSSION

The most commonly identified bacteria overall belonged to the *Leifsonia*, a genus of aquatic bacteria commonly found in water. Although the particular species was not identified, *Leifsonia aquatica*, a bacterium belonging to the *Leifsonia* genus, causes catheter-related disease and, in rare cases, acute sepsis in immunocompromised patients (9,10). The next most commonly identified genus, *Methylobacterium*, comprises opportunistic pathogenic bacteria that cause infections in immunocompromised individuals (11). Streptococcus, the third most commonly identified genus, is composed of gram-positive bacteria found in large numbers in the oral cavity and saliva, attaching to the oral mucosa and the surfaces of teeth (12). The fourth most common, *Haemophilus*, includes life-threatening microorganisms that cause respiratory infection and are known to have wide pathogenicity (13).

Table I
List of all bacterial genera identified in lipstick using pyrosequencing

	Phylum	Class	Genus	Number
Bacteria	*Acidobacteria*	*Solibacteres*	*Paludibaculum*	1
	Actinobacteria	*Acidimicrobiia*	*Acidimicrobiaceae_uc*	1
		Actinobacteria_c	*Actinobacteria_c_uc_g*	2
			Actinomyces	42
			Actinomycetaceae_uc	1
			Scardovia	1
			Corynebacteriaceae_uc	1
			Corynebacterium	12
			Mycobacterium	5
			Rhodococcus	2
			Calidifontibacter	15
			Arsenicicoccus	4
			Janibacter	2
			Agromyces	1
			Cnuibacter	1
			Diaminobutyricibacter	2
			Leifsonia	13,081
			Microbacteriaceae_uc	276
			Rothia	120
			Micrococcales_uc_g	13
			Propionibacterium	12
		Coriobacteriia	*Atopobium*	2
		Rubrobacteria	*Gaiellaceae_uc*	1
	Bacteroidetes	*Bacteroidia*	*Bacteroides*	2
			Bacteroidales_uc_g	1
			Porphyromonas	8
			Alloprevotella	1
			Prevotella	23
		Flavobacteria	*Capnocytophaga*	4
			Chryseobacterium	5
			Elizabethkingia	3
		Sphingobacteriia	*Sphingobacteriia_uc_g*	1
	Chloroflexi	*Caldilineae*	*Caldilineaceae_uc*	1
	Deinococcus-Thermus	*Deinococci*	*Deinococcus*	3
			Thermus	4
	Firmicutes	*Bacilli*	*Paenibacillaceae_uc*	1
			Staphylococcaceae_uc	2
			Staphylococcus	59
			Gemella	16
			Granulicatella	7
			Lactobacillus	9
			Lactobacillales_uc_g	1
			Leuconostoc	2

Table I
Continued

Phylum	Class	Genus	Number
		Lactococcus	12
		Streptococcaceae_uc	24
		Streptococcus	1,492
	Clostridia	*Clostridiales_uc_g*	1
		Blautia	2
		Lachnoanaerobaculum	1
		Lachnospiraceae_uc	1
		Shuttleworthia	12
		Eubacterium_g11	7
		Faecalibacterium	1
		Ruminococcaceae_uc	3
	Negativicutes	*Dialister*	6
		Selenomonas	2
		Veillonella	34
Fusobacteria	*Fusobacteria_c*	*Fusobacterium*	13
		Leptotrichia	28
Nitrospirae	*Nitrospira_c*	*Nitrospira*	1
Planctomycetes	*Planctomycetacia*	*Planctomycetacia_uc_g*	1
		Planctomycetaceae_uc	2
Proteobacteria	*Alphaproteobacteria*	*Caulobacter*	7
		Bradyrhizobium	45
		Devosia_f_uc	1
		Bosea	1
		Methylobacteriaceae_uc	35
		Methylobacterium	2,969
		Rhizobiaceae_uc	1
		Rhizobiales_uc_g	2
		Rhizomicrobium_f_uc	1
		Rhodospirillales_uc_g	3
		Rickettsiaceae_uc	1
		Altererythrobacter	1
		Sphingomonadaceae_uc	2
		Sphingomonas	36
	Betaproteobacteria	*Rhodoferax*	1
		Derxia_f_uc	1
		Lautropia	46
		Kingella	1
		Neisseria	189
		Neisseriaceae_uc	3
		Simonsiella	4
		Dechloromonas	2
		Zoogloea	1

Table I
Continued

	Phylum	Class	Genus	Number
		Gammaproteobacteria	*Cardiobacterium*	10
			Enterobacter	7
			Escherichia	13
			Salmonella	2
			Trabulsiella	1
			Halomonas	5
			Oceanospirillales_uc_g	1
			Haemophilus	712
			Pasteurellaceae_uc	16
			Acinetobacter	5
			Moraxella	27
			Moraxellaceae_uc	2
			Pseudomonadaceae_uc	5
			Pseudomonas	190
			Pseudomonadales_uc_g	1
			Lysobacter	4
			Xanthomonadaceae_uc	1
			Xanthomonadales_uc_g	1
	Saccharibacteria_TM7	*Saccharimonas_c*	*Saccharimonas*	9
	Verrucomicrobia	*Verrucomicrobiae*	*Pedosphaera_f_uc*	2
Unkown				102
Total				19,863

Repeated use of cosmetics can cause microbial contamination and infection (14). In general, cosmetics should be free from the following bacterial species: *S. aureus*, *E. coli*, *Salmonella* spp., *C. albicans*, *Clostridium* spp., and *P. aeruginosa* (3). *Staphylococcus* and *Pseudomonas* were identified in this experiment, whereas pathogenic bacteria such as *Escherichia* and *Salmonella* were found to spread through the fecal-oral route. In addition, oral bacteria associated with disease, such as *Actinomyces*, *Porphyromonas*, and *Fusobacterium* were found. Pathogenic bacteria such as *Corynebacterium* and *Neisseria* were also found in this study. In particular, *Corynebacterium diphtheriae* of the *Corynebacterium* genus is the cause of diphtheria (15). *Neisseria meningitidis* and *Neisseria gonorrhoeae* of the *Neisseria* genus cluster on the mucosal surfaces of humans. The former is known to cause sepsis and meningitis, whereas the latter causes gonorrhea (16). As our sequencing was not deep enough to identify the particular species present, additional studies will be needed to determine whether these pathogenic bacteria are present in lipstick. In addition, a genus was considered pathogenic if any one of its members were known pathogens. Therefore, the actual proportion of pathogenic bacteria might be lower than reported here.

Previous studies examining bacterial contamination of lipstick using classical bacterial cultures have reported contamination by various species. Onondaga et al. conducted a Gram stain and biochemical test on 20 lipstick samples (3) and observed contamination by *S. aureus* and *C. albicans*. Sawant et al. examined 12 lipstick samples for contamination before and

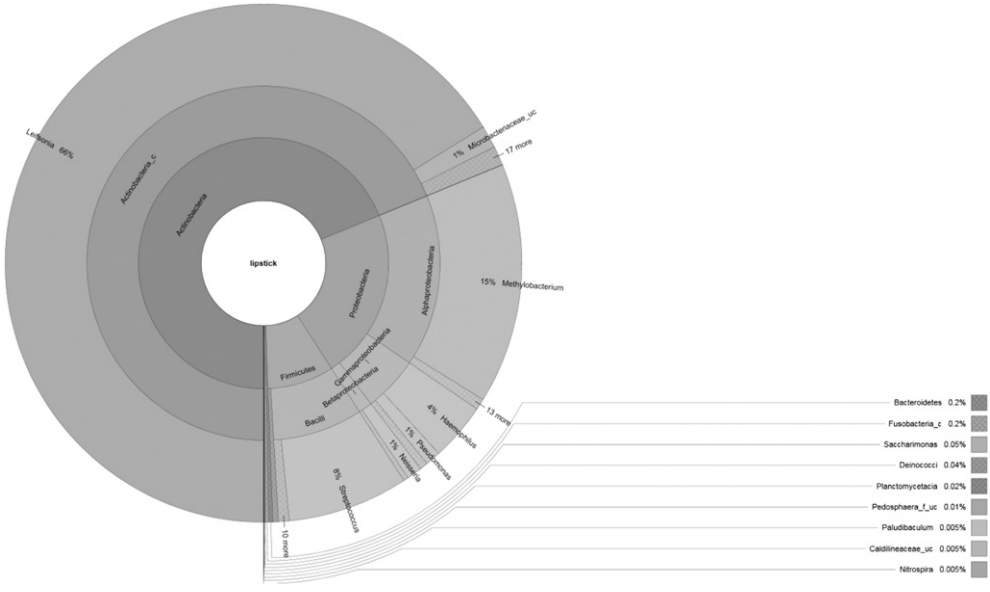

Figure 1. Diversity of the bacterial community present in lipstick samples.

after use (4). *Proteus*, *Providencia*, *Morganella*, *Staphylococcus*, and *Pseudomonas* were detected using 16S rDNA sequencing, Gram stain, and biochemical characterization. Although these studies are limited to culturable bacteria, they have nonetheless detected the presence of various genera of bacteria on lipstick.

Preservatives are commonly used to ensure the stability and safety of cosmetic products (14,17). Various preservatives have been used to maintain low levels of microorganism contamination and to increase the shelf life of lipstick (4,18). Air contact following opening of the cap has been reported to increase microbial contamination, although preservatives possess sufficient antimicrobial activity to maintain product safety (4). In our study, 20 lipstick samples were plated on a blood agar plate and cultured in a 5% CO_2 incubator for 48 h. Live bacteria were found in seven of 20 samples (data not shown). These results suggest that the antiseptics contained in lipstick may be present in insufficient concentrations for antimicrobial activity resulting in contamination.

In this study, we investigated the diversity of contaminating bacteria in lipstick using pyrosequencing. We detected a wider diversity of contaminating bacteria compared with previous studies. Lipstick comes into direct contact with the mouth, and because it is reused, microorganisms can infect the skin as well. We suggest that consumers should use products that inhibit the growth of contaminating bacteria on their cosmetics, and that the types of preservatives as well as their concentrations should be optimized.

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