Temperature regulates bacterial protein production: Possible role in rosacea

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Facial skin temperature is higher for patients with rosacea. Papules and pustules might arise because bacteria behave differently at these warmer temperatures. We sought to: (1) compare bacteria from facial skin of patients with rosacea with that of control subjects; and (2) grow these bacteria at 30°C and 37°C to compare growth curves and secreted proteins. Bacteria isolated from pustules/skin surfaces of patients with rosacea and skin surfaces of control subjects were identified and cultured at 37°C and 30°C. Secreted proteins were separated by electrophoresis. We found that *Staphylococcus epidermidis* isolated from patients with rosacea was consistently β-hemolytic, whereas that from control subjects were nonhemolytic. Bacteria from patients with rosacea grew at the same rate and to the same stationary phase whether cultured at 37°C or 30°C. Isolates from patients with rosacea secreted more proteins, and generally more of each protein at 37°C compared with 30°C. In conclusion, bacteria isolated from patients with rosacea secrete different proteins and different amounts of protein at different temperatures. (J Am Acad Dermatol 2004;50: 266-72.)

Rosacea is a syndrome consisting of: (1) persisting vivid red centrofacial erythema; (2) flushing; (3) telangiectases; (4) stinging and burning sensations; (5) mild facial eczematous dermatitis; (6) ocular rosacea; (7) phymas; and (8) centrofacial papules, pustules, or both. Pustules often appear suddenly in groups, often on a red skin base. Aerobic cultures of pustules often fail to isolate bacteria. Biopsy specimens show no bacteria in follicles,[1] but cultures not infrequently isolate strains of *Staphylococcus epidermidis*. These bacteria normally inhabit the skin, and their isolation is often viewed as contamination or as an incidental finding.

The usual failure to see or isolate unusual microbial organisms or known pathogens suggests that something else besides bacteria causes the pustules of rosacea. Because papules and pustules often develop on red edematous skin, edema has been implicated as the fuse that somehow ignites inflammation.[2] If so, pathogenic mechanisms are uncertain.

On the other hand, papules and pustules are similar to those developing among patients with acne vulgaris, where bacteria (*Propionibacterium acnes*) play a role. For patients with rosacea as in those with acne, papules and pustules often disappear (and fail to develop) when patients are receiving any one of a large number of chemically different antibiotics. These facts suggest that bacteria sensitive to those antibiotics might directly or indirectly induce the inflammation and elicit papules and pustules.

Facial skin temperature is higher among patients with rosacea compared with healthy persons as a consequence of the persisting facial erythema and flush. The higher blood flow through facial skin of patients with rosacea and the closeness of vessels to the skin leads to higher skin surface temperatures. Because bacteria can grow at different rates, and secrete different toxins and other products at different temperatures,[3] papules and pustules might arise because bacteria behave differently at these warmer temperatures. Members of the usually nonpathogenic normal skin flora such as *S epidermidis* may replicate at different rates at different temperatures, or they might make and secrete more or different virulent proteins at different temperatures.

In this study, we show that *S epidermidis* strains isolated from the skin of patients with rosacea were different from bacteria isolated from control subjects, and we show that they produce more proteins at 37°C than at 30°C.

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METHODS

Isolation of bacteria

Isolates were obtained from 4 patients with untreated rosacea. A pustule was incised with a No. 11 blade, and purulent contents were extruded from the pustule by gentle squeezing. The pus from 1 or several pustules was collected on a cotton-tipped applicator and transported to the laboratory in transport media. To isolate bacteria, swabs were streaked onto sheep blood agar plates and incubated both aerobically and anaerobically. After 24 hours, the plates were examined. Swab samples were also obtained from the skin surface of 4 control subjects without rosacea.

Growth kinetics

All organisms were cultured in 100 mL of a dialyzable beef heart medium with shaking (200 rpm) at both 30°C and 37°C until stationary growth phase was achieved. Plate counts were used to assess the growth kinetics of the organisms at both temperatures at 0, 2, 4, 6, 8, and 24 hours.

Protein secretion

After 24 hours, cultures were treated with 4 volumes of absolute ethanol to precipitate secreted proteins (4°C). The precipitates were collected by centrifugation, dried, and suspended to 1/100 the original volume in distilled water. The insoluble material was removed by centrifugation (13,000*g*, 10 minutes). The samples were then mixed 50:50 with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer and electrophoresed in 10% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R 250, and protein sizes were estimated by comparison with molecular weight standards, also included.

Tributyrin assay

The tributyrin assay detects lipolysis in fluids. Lipases cleave the lipid tributyrin in agarose gels.[4] Briefly, tributyrin was suspended in phosphate-buffered saline (0.5% vol/vol) and added to melted agarose to obtain a final agarose concentration (vol/vol) of 0.75%. The solution was vortexed to form a white emulsion, and layered onto microscope slides (2 mL/slide). After cooling to allow the agarose to solidify, 4-mm diameter wells were cut out of the agarose, and 10 μL of samples were added to the wells. Clearing of the tributyrin emulsion after overnight incubation at 37°C in a humidified chamber indicated hydrolysis (lipolysis).

Assessment for hemolytic activity

Hemolytic activity was assessed by 2 methods. First, bacteria were cultured on sheep blood agar plates and observed for hemolysis. Second, rabbit erythrocytes (0.1% vol/vol) were suspended in 0.75% agarose in phosphate-buffered saline, and 2 mL/slide used to coat microscope slides. After the agarose solidified, 4-mm diameter wells were cut into the agarose, and 10 μL of culture supernate added to wells. The slides were then incubated overnight in a humidified chamber at 37°C and examined for hemolysis of the rabbit erythrocytes.

Time-course studies

To determine when the lipase was produced, isolated coagulase-negative staphylococci were grown at 37°C with shaking in 100 mL of dialyzable beef heart medium. Samples (10 mL) were removed at 2, 4, 6, 8, and 24 hours, and standard plate counts were done. The remainders of the samples were treated with 4 volumes of ethanol to precipitate proteins. The precipitates from each sample were collected individually and resuspended in water (1/100 original volume). Insoluble material was removed by centrifugation, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was stained with Coomassie Brilliant Blue R 250. To estimate the amounts of secreted lipase protein, bands were scanned densitometrically. Band density was graphed versus growth.

In other experiments coagulase-negative staphylococci (strain MNCh) were cultured in dialyzable beef heart medium for 4, 8, and 12 hours in the presence and absence of glycerol monolaurate at 37°C with shaking. Bacterial numbers were estimated by plate counts at each time point and lipase activities were quantified by the tributyrin assay described above.

RESULTS

Isolation of bacteria

All isolates from patients contained pure cultures of staphylococci. In one patient 2 different staphylococci were obtained; only 1 of these was culturable in batch beef heart medium (see below) and for this patient only this strain was used for further experiments. Staphylococci also were isolated from all 4 control subjects.

Microbiology

All isolated bacteria from patients and control subjects were coagulase-negative *S epidermidis*. The staphylococci from patients with rosacea were consistently β-hemolytic when cultured on blood agar

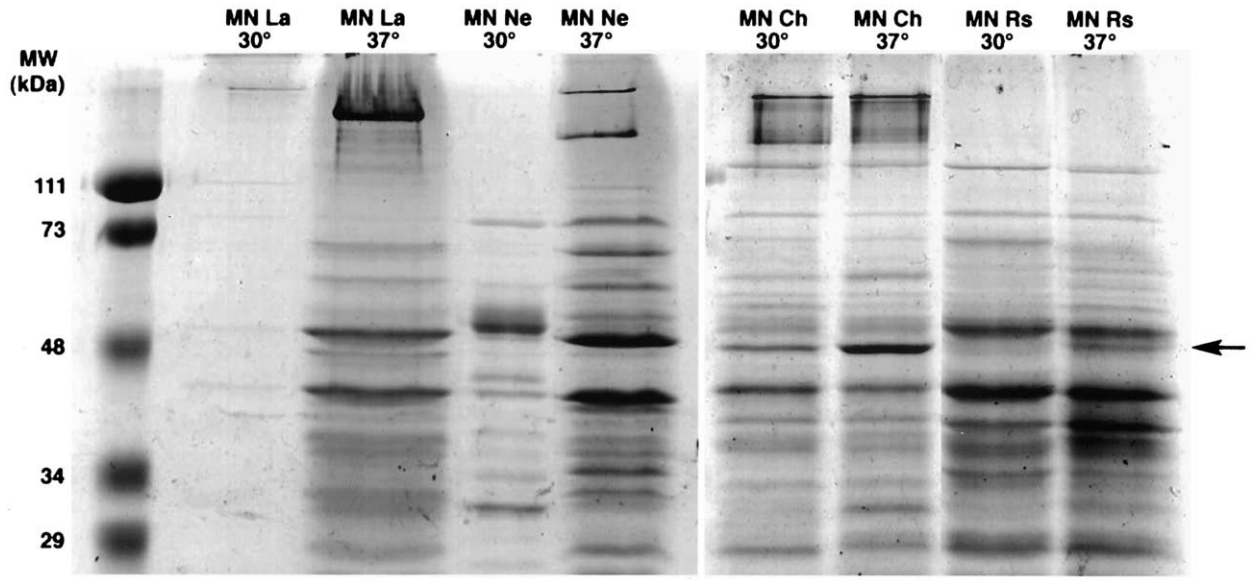

Fig 1. Exoprotein production by 4 strains of *Staphylococcus epidermidis* isolated from patients with rosacea after culture at 30°C and 37°C as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Molecular weights (*MW*)(in kilodaltons [*kDa*]) indicate molecular size of MW standards included in *lane 1*. Rosacea strains: MN La; MN Ne; MN Ch; and MN Rs. *Arrow*, Location of lipase protein sequenced from strain MN Ch.

plates, whereas the staphylococci from control subjects were nonhemolytic.

Bacteria from patients with rosacea grew at the same rate whether cultured at 37°C or 30°C (Fig 1). They also grew to the same stationary phase. Thus, the difference in production of secreted proteins was not the result of different growth rates.

All 4 organisms from patients with rosacea secreted significantly more proteins at 37°C compared with 30°C, and all 4 secreted significantly more of most individual shared proteins at both temperatures. Two proteins were most often very highly expressed at 37°C compared with 30°C (Fig 2). These 2 proteins were subjected to further analysis. However, many other proteins were also made at higher concentrations at 37°C than at 30°C. These were not analyzed further.

The higher molecular weight protein (Fig 2) in the 2 examples had a molecular size of 45 kd. The lower molecular weight protein had a size of 28 kd. A second set of sodium dodecyl sulfate polyacrylamide gel electrophoresis experiments were performed. Proteins were transferred to polyvinylidine difluoride membranes (Bio-Rad Laboratories, Hercules, Calif). These membranes were briefly stained with Coomassie Brilliant Blue to locate the bands, and then destained. The membrane regions corresponding to each of the 2 protein bands were excised from the membrane and sent to the Mayo Clinic Rochester for N-terminal protein sequencing (10 residues). The dominant band containing the

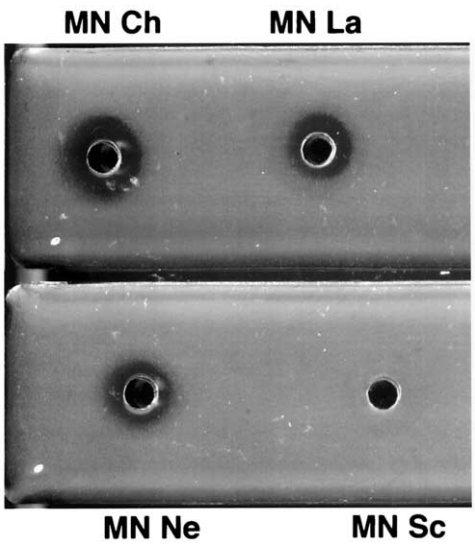

Fig 2. Tributyrin lipase activity of rosacea strains of *Staphylococcus epidermidis*. Rosacea strains: MN Ch; MN La; and MN Ne. Nonrosacea strain: MN Sc. Sequence indicates lipase demonstrated to be produced by MN Ch strain. First 10 boxed amino acids indicate residues that were identified by sequencing.

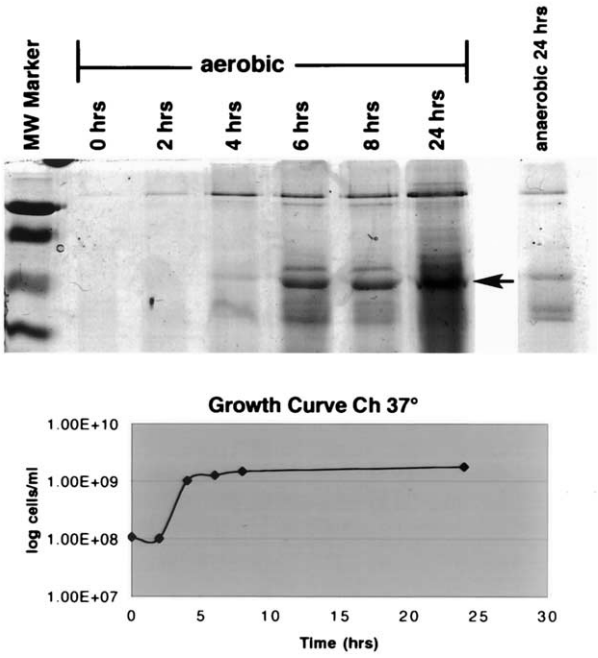

Fig 3. Time course for production of lipase by rosacea *Staphylococcus epidermidis* MN Ch. *Lane 1* shows molecular weight (^{MW}) markers. Aerobic lanes contained proteins from 0-, 2-, 4-, 6-, 8-, and 24-hour incubations in air. Anaerobic culture for 24 hours shown in *lane 8* contained band of slightly higher MW than lipase that did not demonstrate lipase activity. Time course for growth of MN Ch is shown in *lower part of figure*. Inoculum size was approximately 10^8/mL; stationary phase was reached by 5 to 6 hours postinoculation. *Arrow*, Location of lipase protein.

higher molecular weight protein was identified as staphylococcal lipase as previously cloned and sequenced[5] (Fig 3). The lipase made by these strains was not expressed under anaerobic conditions. The second band gave no sequence, so its identity remains unknown.

Tributyrin assay

To prove its lipase action, supernates of bacterial cultures were added to gels containing tributyrin. All of the rosacea isolates from patients with rosacea made significant amounts of lipase as detected by this assay, but only half of the control isolates made this lipase protein.

Time-course studies

A time-course experiment was used to determine when the lipase was made. Most protein was made in the late logarithmic phase (postexponential phase) as organisms were beginning to encounter nutrient depletion (Fig 2).

In a second study, the effect of glyceryl monolaurate on production of lipase by *S epidermidis* MNCh was determined over a 24-hour period. Glycerol monolaurate was previously shown to inhibit production of exotoxins by gram-positive cocci.[6,7] Glycerol monolaurate inhibited the production of lipase by a representative *S epidermidis* strain from one of the patients with rosacea (Table I). Glycerol monolaurate was highly effective in inhibiting lipase production by the strain at subgrowth inhibitory concentrations. At 50 μg/mL, glycerol monolaurate inhibited both the growth of the organism and the production of lipase.

DISCUSSION

This is a pilot study investigating the effect of temperature on the growth of bacteria isolated from the skin of patients with rosacea. We showed that bacteria isolated from the skin of patients with rosacea behave differently when cultured at 37°C compared with 30°C.

The surface skin temperature of patients with rosacea is relatively elevated. In vitro, isolated bacteria from patients with rosacea produced many more proteins at 37°C than they did at 30°C. For most of those proteins secreted at both temperatures, the rates of secretion of various proteins were also higher at the higher temperature. Furthermore, genes governing production of several proteins showed consistent up-regulation for all isolates.

The papules and pustules of rosacea often disappear quickly when patients receive topical and systemic antibiotics. For example, all papules and pustules disappeared within 3 months in 59% of patients with moderate to severe rosacea treated with systemic tetracycline and topical metronidazole gel.[8] Even among the 41% of patients receiving treatments who did not stop developing pustules within 3 months, the numbers of pustules in those patients decreased markedly. Similar improvement rates result when patients are treated with any one of an array of chemically dissimilar antibiotics including erythromycin, clindamycin,[9] ampicillin,[10] metronidazole,[11] clarithromycin,[12] and sulfa medications. The abolition of papules and pustules for patients with rosacea treated with antibiotics has been attributed to their anti-inflammatory activity. This mechanism seems unlikely because: (1) antibiotics abolish the lesions completely rather than just blunting the inflammatory reaction; (2) agents with much more potent anti-inflammatory activity such as topical steroids and nonsteroidal anti-inflammatory drugs are not routinely as effective; (3) most antibiotics work well

Table I. Effect of glycerol monolaurate on *Staphylococcus epidermidis* MNCh growth and lipase production

Glycerol monolaurate (μg/mL)	Incubation time (hours)					
	4		8		24	
	Log cells/mL*	Lipase†	Log cells/mL*	Lipase†	Log cells/mL*	Lipase†
0	6.7	18	9.6	20	9.5	22
10	5.8	4	9.2	6	9.6	11
20	6.0	0	8.8	0	9.3	6
50	5.8	0	5.6	0	5.9	0

*Inoculum size 1.3×10^6/mL.
†Lipase units measured as millimeter diameter of tributyrin hydrolysis.

despite divergent chemical structures and alleged mechanisms of anti-inflammatory activity; (4) antibiotics do not completely abolish most other inflammatory dermatoses and often do not even hint at clinically relevant anti-inflammatory activity; and (5) there is no evidence to implicate free radical formation as a cause (allegedly quenched by antibiotics such as metronidazole).[13]

Despite failure to isolate unusual skin microbes by culture of pustules and skin, the dramatic disappearance of papules and pustules for patients treated with such chemically different antibiotics suggests that bacteria do, indeed, play a role in pathogenesis. Facial skin of patients with rosacea is clinically hotter than the facial skin of control subjects, especially during flushing attacks. This is a consequence of increased blood flow through blood vessels on the central face.[14] This means that facial bacteria on the skin surface and in follicles live at abnormally high temperatures, and we found that this influences the nature of the bacteria and the products they produce.

All isolated bacteria from patients and control subjects were coagulase-negative staphylococci, specifically *S epidermidis*. However, the staphylococci from patients with rosacea were consistently β-hemolytic when cultured on blood agar plates, whereas the staphylococci from control subjects were nonhemolytic. Many additional isolates from patients with rosacea and control subjects will be needed to establish whether of not this finding is a constant one.

Bacteria behave differently at different temperatures. Growth rates could have been different, but it turned out they were not. The coagulase-negative staphylococci isolated from patients with rosacea grew at the same rate at 30°C as they did at 37°C. Furthermore, they grew to the same stationary phase density.

Bacteria make different products at different temperatures. The nature and amounts of these proteins may be important. For example, toxic shock syndrome toxin-1 is produced by *S aureus* only at certain specific temperatures.[3] In experiments detailed herein, *S epidermidis* isolated from patients with rosacea made much more protein at 37°C than at 30°C. In addition, supernates of cultures of *S epidermidis* cultured at 37°C produced different proteins and different amounts of individual proteins compared with supernates produced at 30°C.

For all 4 organisms tested, 1 protein was highly up-regulated at 37°C and was a dominant protein on all gels. This protein was isolated, sequenced, and determined to be a major lipase, identical to that previously described.[5] Its role, if any, in rosacea is speculative, but its presence provides a surrogate marker for bacterial virulence and protein production. The lipase gene was positively up-regulated by elevated temperature and the presence of oxygen (data from this study). It was late logarithmic phase–regulated, ie, the lipase was predominantly made when the organisms were beginning to encounter nutrient depletion. Lipase production was inhibited by glycerol monolaurate, a compound previously shown to inhibit exoprotein production by gram-positive cocci, including *S aureus* and *S hominis*. The presence of this lipase may indicate that these strains of *S epidermidis* isolated from patients with rosacea are more virulent or secrete virulence factors. The direct regulator of secreted virulence factors is RNA III termed "agr." This gene also encodes δ-hemolysin (causing the β-hemolysis by the isolates). Secretion of other virulence proteins is expected among bacteria containing agr.[15]

Perhaps other proteins are more important than the lipase. Many other proteins were up-regulated, and each of these should be identified and studied for physiologic effects. Some proteins were detected in supernates from cultures at 37°C that were not seen in supernates from cultures at 30°C. These proteins may be uniquely synthesized at only the

higher temperature and also may be uniquely pathogenic.

Although our studies focused on *S epidermidis*, we are not at all sure *S epidermidis* has relevance to rosacea. It is likely that skin temperature affects the activities of other members of the cutaneous microflora including other aerobic bacteria, anaerobic bacteria, *Demodex folliculorum* and *D brevis*, bacteria in the gut of *Demodex* species, and yeast such as *Malassezia ovalis*. Our research lays the groundwork for similar studies of each of these organisms. What is relevant is the notion that the microbial microenvironment in follicles or on the skin surface of patients with rosacea is a different microenvironment from that of control subjects, and that this aberration may produce disease.

Bacteria from patients with rosacea were isolated by incision of a pustule and harvesting the extruded pus. Care was taken not to touch the skin surface, but it is possible the isolated bacteria came from the skin surface rather than from the follicle. It may not matter. For example, if the lipase is relevant, it may cleave surface lipids leading to high concentrations of lysed lipids at the follicular orifice. These lipids might elicit papules and pustules there in a way similar to those elicited by degraded facial moisturizers.[16]

Cultures compared growth and protein synthesis at 30°C and 37°C. Actual malar skin temperatures of patients with erythematotelangiectatic rosacea average 32.7°C at ambient temperatures of 21°C to 23°C.[17] After oral challenge with hot beverages or alcohol, facial temperature increases about 2 to 3 degrees to about 34°C to 35°C.[18] We chose to study differences at more extreme temperatures to optimize the chances of finding differences. These temperatures are, however, relevant; because facial skin temperatures of about 30°C and 37°C occur in cold and hot environments.[19]

This is a pilot study, designed to test predictions on the basis of a hypothesis that rosacea is a bacterial disease caused by oddities of bacteria living at higher temperatures on flushed faces. We stipulate that the sample sizes were small, that control isolates were from skin surface whereas rosacea isolates were from pustules. We admit, too, that bacteria isolated from pustules may actually be contaminants from the surface, and that the lipase we discuss might be irrelevant to rosacea. Time and further studies will tell, but none of these apparent flaws negate our hypothesis that bacteria isolated from rosacea skin behave differently at different temperatures.

In summary, rosacea skin is warmer than normal and this could alter microbial ecology. Our studies uncovered differences in the nature and behaviors of bacteria isolated from patients with rosacea compared with control subjects. Furthermore, we found differences in the products and quantities of proteins secreted by given bacteria when cultured at different temperatures. Coagulase-negative staphylococci on the skin or in follicles of patients with rosacea make and secrete proteins that may contribute to inflammation as a consequence of living at higher temperatures. Papules, pustules, and dermatitis could result. Further studies are needed to clarify biologic effects of doses of each secreted proteins, the substrates for the lipase we identified, the effects of various secreted products on cells and skin, and the role of products perhaps also produced preferentially at higher temperatures by other bacteria, yeast, and mites.

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